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THE UNIVERSITY OF ALBERTA

STUDIES OF THE BIOSYNTHESIS OF MENGO VIRUS-SPECIFIC POLYPEPTIDES

by

©

EVA PAUCHA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled STUDIES OF THE BIOSYNTHESIS OF MENGO VIRUS-SPECIFIC POLYPEPTIDES submitted by EVA PAUCHA in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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To my parents

for the opportunities they
have given me and for their
unfailing support from the
beginning.

ABSTRACT

Investigations of the synthesis of Mengo virus-specific polypeptides in L.cells have shown that, as is the case with other picornaviruses, the stable viral gene products are formed by post-translational cleavage of larger precursor proteins. Fourteen virus-specific polypeptides have been identified by SDS-polyacrylamide gel electrophoresis of infected cell lysates, and their molecular weights and kinetic behavior have been determined. The sequence in which the viral polypeptides are synthesized was established from experiments in which pactamycin was used to block the initiation of translation. A cleavage scheme which interrelates all fourteen virus-specific proteins has been defined. A polypeptide has been detected in lysates of infected cells prepared after exposure to amino acid analogues (which block post-translational processing) whose size suggests that it is produced by uninterrupted translation of the entire genome and thus may serve as precursor of all virus-specific polypeptides.

All viral proteins do not appear to be produced in equimolar amounts throughout the replicative cycle of the virus, however, since the value of the molar ratio of capsid to non-capsid protein synthesized increases progressively as a function of hours post-infection. It has been suggested that this apparent asymmetry of translation is due to increasing frequency of premature termination of translation at a site located near the mid-point of the viral mRNA, an event which results in a progressive decrease in the production of non-capsid protein.

Comparative studies on the replication of poliovirus in HeLa cells indicated that the genome of this virus too may be translated asymmetri-

cally late in infection. However, the instability of poliovirus-specific polypeptides made precise quantitation difficult. As a result, the data do not provide unequivocal support for the hypothesis that premature termination occurs during the translation of poliovirus m-RNA.

A crude cell-free protein synthesizing system was developed, the objective being to test the termination hypothesis directly using membrane-bound polysomes from Mengo virus-infected cells as template. Preliminary results indicate that high molecular weight (>125,000) polypeptides are produced in this system and that inhibition of post-translational cleavage by the addition of the protease inhibitor TPCK increases the amount of high molecular weight material detected.

Examination by SDS-polyacrylamide gel electrophoresis of ribosomes from Mengo virus-infected L.cells revealed that little or no virus-specific protein is ribosome-bound in this system.

A particle having an estimated sedimentation coefficient of about 50S was detected during analysis of lysates of Mengo virus-infected cells in linear sucrose density gradients. SDS-polyacrylamide gel analysis showed that the 50S particle is composed of equimolar amounts of the capsid polypeptides ε, α and γ. Since the particle could not be labelled with uridine and was insensitive to RNase treatment it was concluded that it contains no RNA. Results obtained from kinetic studies suggest that the 50S particle is a viral precursor although its precise role in the assembly process remains to be determined.

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LIST OF ABBREVIATIONS

EMC virus	- encephalomyocarditis virus
ME virus	- Maus-Elberfeld virus
FMDV	- foot and mouth disease virus
HRV-1A	- human rhinovirus - 1A
CPV	- cytoplasmic polyhedrosis virus
TYMV	- turnip yellow mosaic virus
vRNA	- viral RNA
cRNA	- complementary RNA
mRNA	- messenger RNA
RI-RNA	- replicative intermediate form RNA
RF-RNA	- replicative form RNA
ds-RNA	- double stranded RNA
VP	- viral protein
NCVP	- non-capsid viral protein
M.W.	- molecular weight
RNA.P α	- RNA polymerase, α subunit
β-Gal	- β-galactosidase
DNA.P	- DNA polymerase
phos	- phosphorylase
BSA	- bovine serum albumin
Fum	- fumarase
LDH	- Lactic dehydrogenase
Chy	- chymotrypsinogen
Myo	- myoglobin
SDS	- sodium dodecyl sulfate

LIST OF ABBREVIATIONS (Continued)

DOC	- deoxycholate
TPCK	- tolylsulfonyl-phenylalanyl-chloromethyl ketone
TLCK	- tolylsulfonyl-lysyl-chloromethyl ketone
PMSF	- phenylmethylsulfonylfluoride
2-ME	- 2-mercaptoethanol
cpm	- counts per minute
rpm	- revolutions per minute
g	- centrifugal force relative to gravity
mA	- milliampere
μCi	- microcurie
μM	- micromolar
pfu	- plaque-forming units
moi	- multiplicity of infection
hr. p.i.	- hours post infection

I. GENERAL INTRODUCTION

Picornavirus Classification

The term picornavirus (pico meaning small) was coined in 1963 by the International Enterovirus Study Group to describe a group of small viruses whose genome consisted of a single strand of ribonucleic acid. The picornaviruses, by definition, are distinguished by the following criteria: (1) their small size (150 to 300 Å in diameter), (2) the absence of an essential lipid envelope, and (3) their single-stranded RNA genomes. Although there are plant and bacterial viruses which meet these criteria, the term has been applied to viruses of animal origin only.

The picornaviruses have been further classified into subgroups on the basis of differences in the pH stability of the virions and in their buoyant densities in solutions of cesium salts (Andrewes and Pereira, 1972; Newman et al, 1973; Scraba and Colter, 1974). The five subgroups distinguishable by these criteria are shown in Table 1.

Although the caliciviruses are included as a subgroup, their larger size and characteristic capsid morphology (Zwillenberg and Bücki, 1966; Almeida et al, 1968; Wawrzkiewicz et al, 1968) are atypical. In addition, viruses in this subgroup appear to possess only one major capsid protein (Bachrach and Hess, 1973; Burroughs and Brown, 1974) while all other picornavirus capsids are composed of four distinct polypeptides (Rueckert, 1971; Fenner et al, 1974). For these reasons, Burroughs and Brown (1974) have proposed that this group of viruses be removed from the family Picornaviridae and be reclassified as a new family of Caliciviridae.

TABLE 1

Picornavirus Classification Scheme

Subgroup	Viruses	Distinguishing Characteristics
Enterovirus	Polio (3 serotypes) Coxsackie A (24 serotypes) Coxsackie B (6 serotypes) Echo (∿ 35 serotypes) Enteroviruses of various animals	Buoyant density (in CsCl) = 1.33-1.35 g/ml Virions stable pH 3-10 Empty capsids produced *in vivo*
Cardiovirus	EMC (Encephalomyocarditis) ME (Maus-Elberfeld) Mengo Columbia-SK	All are antigenically related. Buoyant density = 1.34 g/ml Virions stable at low pH, but dissociate at $5 < pH < 7$ in the presence of Cl^- or Br^- ions No stable empty capsids produced *in vivo*
Rhinovirus	Human rhinovirus (∿ 90 serotypes) Equine and bovine rhinoviruses	Buoyant density = 1.38-1.45 g/ml Virions dissociate at $pH < 5$ Some varieties produce empty capsids *in vivo*
FMDV	Foot-and-mouth disease virus (7 serotypes)	Buoyant density = 1.43 g/ml Virions dissociate at $pH < 6.5$ Empty capsids produced *in vivo*
Calicivirus	Vesicular exanthema (pigs) Feline calicivirus San Miguel sea lion virus	Buoyant density = 1.37 g/ml Virions dissociate at $pH < 4$ Slightly larger (35-40 nm diam.) than the above viruses; show cup-like capsomeres when negatively stained

Modified from Scraba and Colter, 1974. The various picornavirus subgroups also have distinctive pathological properties. These are discussed in detail by Andrewes and Pereira (1972). Echo is an abbreviation for "enteric cytopathic human orphan".

Properties of the Virion

Picornaviruses in general and Mengovirus in particular are isometric particles with hydrated diameters of about 300 Å. The virions contain approximately 70% protein and 30% RNA by weight (Rueckert, 1971; Fenner et al, 1974; Scraba and Colter, 1974). They have sedimentation coefficients ($S^{o}_{20,w}$) in the range of 150-160 S, diffusion coefficients ($D^{o}_{20,w}$) of 1.44-1.47 x 10^{-7} cm^2/sec and partial specific volumes ($\bar{V}$) of 0.68-0.70 ml/g (Scraba et al, 1967; Rueckert, 1971; Scraba and Colter, 1974). By substituting these values in the Svedberg equation, a molecular weight of 8.3-8.5 x 10^6 may be calculated. (Scraba et al, 1967; Rueckert, 1971; Scraba and Colter, 1974).

The Viral RNA

Infectious RNA has been extracted from the enteroviruses polio (Alexander et al, 1958) and Coxsackie (Mattern, 1962), from the cardioviruses Mengo (Colter et al, 1957), EMC (Huppert and Sanders, 1958) and ME (Franklin et al, 1959), and from FMDV (Bachrach et al, 1964). There is nothing unusual about the base compositions of picornaviral genomes. The entero- and cardiovirus RNAs contain approximately equimolar amounts of all four bases, while those of the rhinoviruses differ in that they appear to possess somewhat lower amounts of guanylate (Newman et al, 1973; Rueckert, 1974). None have been found to contain any modified bases.

Until recently, the accepted molecular weight of picornaviral RNAs was about 2 x 10^6 (1.8 to 2.8 x 10^6). Reexamination of these values by newer techniques has resulted in more precise estimates: 2.56 x 10^6 for polio (Tannock et al, 1970), 2.7 x 10^6 for EMC (Burness,

1970) and 2.44 x 10^6 for Mengo (Ziola and Scraba, 1974).

Stretches of polyadenylic acid (poly A), covalently linked to the 3' end of the molecule, have been found in a number of viral and cellular mRNAs (Brawerman, 1974). Poly A stretches 50 to 100 nucleotides in length have been found in the RNAs of poliovirus (Yogo and Wimmer, 1972; Spector and Baltimore, 1975a), Columbia-SK virus (Johnston and Bose, 1972) and rhinovirus (Nair and Owens, 1974; McNaughton and Dimmock, 1975). The cardioviruses EMC (Gillespie et al, 1973; Porter et al, 1974; Burness et al, 1975) and Mengo (Miller and Plagemann, 1972; Spector and Baltimore, 1975b) also contain poly (A) stretches but there is some uncertainty to their length. The function of this feature is unknown, but it appears to be essential for the infectivity of isolated viral RNA (Burness et al, 1975). The presence of A-rich segments has been correlated to messenger function (Johnston and Bose, 1972). It has also been suggested that the presence of poly A may be required for encapsidation of the viral RNA (Spector and Baltimore, 1975b).

Little is known about the 5' end of the picornaviral RNAs. They apparently lack the $m^7G(5')ppp(5')Nmp...$ cap (Wimmer et al, 1975) recently found to be associated with the mRNAs of reovirus, VSV (Muthukrishnan et al, 1975) CPV and vaccinia virus (Furuichi et al, 1975) and with messengers from rabbit reticulocytes (Muthukrishnan et al, 1975) and from mouse myeloma cells (Adams and Cory, 1975), the presence of which is required for translation of the mRNAs in a cell-free system (Muthukrishnan et al, 1975).

The cardioviruses Mengo and EMC, as well as FMDV, contain tracts of poly (C) in their RNAs (Brown et al, 1974; Porter et al, 1974). It has been suggested that these may act as recognition sites for the

viral RNA replicase in a manner analogous to that proposed for the phage Qβ (Küppers and Sumper, 1975), a suggestion compatible with the observation that a partially purified RNA polymerase isolated from EMC virus-infected cells will synthesize poly (rG) on a poly (rC) template (Rosenberg et al, 1972). However, no poly (C) tracts have been found in the RNAs of members of the entero- and rhinovirus subgroups (Brown et al, 1975), an observation which casts some doubt on the proposed function of this structural feature, since the replication of all picornaviruses appears to proceed by essentially the same mechanism.

The Virion Proteins

Amino acid composition analyses of total protein from a number of picornaviruses have been reported. The results, which have been tabulated by Rueckert (1971), show a marked similarity, the only remarkable features of picornaviral proteins being the low content of sulfur-containing residues (2-3 moles %) and the unusually high proline content (6-8 moles %). These proteins contain relatively large amounts of other non-α-helix forming residues (serine, threonine and glycine) in agreement with the estimate, based on optical rotatory dispersion and circular dichroism measurements, that Mengo-virus proteins in situ contain less than 5% α-helix (Scraba et al, 1967; Kay et al, 1970).

The results of ultracentrifugal analyses of picornaviral capsid proteins suggested that each virus contained a single polypeptide species of molecular weight in the range of 26,000 to 30,000 (Maizel, 1963; Rueckert, 1965; Burness and Walters, 1967). However, subsequent analyses by electrophoresis on polyacrylamide gels in the presence of

urea showed that the protein extracted from poliovirus (Maizel, 1963) and from ME-, EMC and Mengo-viruses (Rueckert, 1965; Rueckert and Duesberg, 1966) contained several distinct electrophoretic components. With the discovery that the electrophoretic mobility of a polypeptide in polyacrylamide gels containing sodium dodecyl sulfate (SDS) is directly proportional to the logarithm of its molecular weight (Shapiro et al, 1967; Weber and Osborn, 1969; Dunker and Rueckert, 1969) it became possible to determine simultaneously the number, the molecular weights and the relative amounts of the polypeptide chains in the virions. Thereafter, it was rapidly established that both poliovirus (Maizel and Summers, 1968) and ME-virus (Dunker and Rueckert, 1969) capsids are composed of four polypeptides with molecular weights approximately 34,000, 29,000, 25,000 and 6,000 to 10,000. To date, it has been shown that the capsids of all picornaviruses examined (with the exception of the caliciviruses mentioned previously) are composed of approximately 60 copies of each of four polypeptides of molecular weights close to those cited above (Scraba and Colter, 1974).

Morphology and Architecture of the Virion[1]

With the purification and crystallization of poliovirus (Schaffer and Schwerdt, 1955 and 1959) it became possible to probe picornaviral structure by X-ray crystallography. Finch and Klug (1959) examined crystals of poliovirus by this method and concluded that the virus possesses icosahedral (5:3:2) symmetry, implying that the capsid is constructed of 60 (or 60n) identical asymmetric structure units (Caspar, 1956; Caspar and Klug, 1962). From the regular modulation of

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[1]For review, see Rueckert, 1971.

intensity in the diffraction patterns they suggested that the structure unit has a diameter of 60 to 65 Å. Some doubt was cast on this conclusion, however, when electron microscopy showed that turnip yellow mosaic virus (TYMV), which produces an X-ray diffraction pattern virtually identical to that of poliovirus (Klug et al, 1957), possesses 32 capsomeres, 60 Å apart (Klug and Finch, 1960). Klug and Finch concluded that the 180 identical polypeptide chains which form the TYMV capsid (Harris and Hindley, 1961) are quasi-equivalently clustered into 12 pentamers and 20 hexamers (Klug et al, 1966; Finch and Klug, 1966).

Attempts to determine the number of capsomeres present in picornaviruses by electron microscopic examination of negatively stained preparations have been unsuccessful since the capsids of these viruses are extremely compact and essentially impermeable to the heavy metal salts commonly used as negative stains. The conflicting reports of 32-(Mayor, 1964), 42-(Agrawal, 1966) and 60-(Horne and Nagington, 1959) capsomeres per virion have been based on the appearance of one or two "favorably" stained and oriented, and thus non-representative, particles in a field containing a large number of virions. The relative positions of four distinct polypeptide species must also be considered in any model of picornaviral architecture.

These problems were resolved in part by Rueckert and his coworkers who studied the products of controlled dissociation of ME-virus capsids (Rueckert et al, 1969; Dunker and Rueckert, 1971). It has been shown that the cardioviruses ME (Rueckert et al, 1969; Dunker and Rueckert, 1971), EMC (McGregor et al, 1975), and Mengo (Mak et al, 1970) are inactivated by incubation at pH 5.5 to 7.0 in the presence of 0.1 to

0.2 M chloride ions. This inactivation is caused by dissociation of the viral capsid into subunits (sed.coeff. ≈ 14S; M.W. ≈ 425,000) with the concomitant release of the viral RNA. Rueckert et al (1969) showed that the 14S subunit may be further dissociated by incubation with 1-2 M urea into a product having a sed.coeff. of 5S and M.W. of about 86,000. They also demonstrated, by SDS-polyacrylamide gel analyses, that both fragments contain equimolar amounts of the capsid polypeptides α, β and γ. On the basis of these data, these authors proposed a 60 subunit model for the structure of ME-virus, in which the basic structure unit is a protomer composed of one molecule of each of the non-identical polypeptides α, β and γ (i.e. the 5S protomer). It is envisioned that these structure units associate in clusters of five to form the 14S subunits (pentamers), one of which is centered at each vertex of the icosahedral particle (Dunker and Rueckert, 1971).

Supporting evidence for this model has been provided by similar studies of Mengo virus (Mak et al, 1974). Dissociation of the Mengo virion at pH 6.2 results in the production of a 13.4S subunit which can be further broken down by urea into 4.7S fragments. SDS-polyacrylamide gel electrophoresis has confirmed that the polypeptide composition of both dissociation products is the same as that of the corresponding ME virus fragments; that is, both contain equimolar amounts of the capsid polypeptides α, β and γ. The dimensions of both the 13.4S and the 4.7S fragments, as determined by electron microscopy, are consistent with the notion that the 4.7S is the fundamental structure unit of the capsid and that the 13.4S is a pentameric cluster of these units. These authors believe that the virion is composed of 60-4.7S units

(whose diameter of 68 Å agrees well with the original prediction of Finch and Klug, 1959) arranged in 12 groups of 5 units each (the 13.4S pentamers). One such pentamer would be located at each vertex of the icosahedron, as proposed by Dunker and Rueckert (1971).

There is still some uncertainty regarding the fine detail of picornavirus capsid architecture. It is not known, for example, whether the three non-identical polypeptides which make up the 4.7S unit are located at specific sites within the unit or whether they exist as an interwoven "trimeric" complex. The true situation may be somewhere in between. The relationship of the smallest capsid polypeptide, δ, to the 4.7S unit is also unknown. Mak et al (1974) have proposed that this protein is clustered in trimers, one of which is located on each of the 20 faces of the icosahedral Mengo virion. On the other hand, it has been proposed that this polypeptide occupies an internal site in bovine enterovirus (Johnston and Martin, 1971) and FMDV (Talbot et al, 1973), while Philipson et al (1973) favor a model for Coxsackie B3 in which this protein is located (as pentamers) at the vertices of the icosahedron. Although the capsid architecture of all picornaviruses is basically similar, it seems quite likely that differences in fine structure may exist among the various subgroups.

Viral Replication[1]: Early Interaction Between Cells and Virus[2]

Attachment

To initiate infection, virus particles attach to specific receptors

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[1]For general review, see Baltimore, 1969 and Fenner et al, 1974.

[2]For a comprehensive review of this subject, see Lonberg-Holm and Philipson, 1974.

located in the membrane of susceptible cells. It has been estimated that each cell possesses 10^4 to 10^5 receptors of a particular type (Crowell et al, 1971; Lonberg-Holm and Korant, 1972). These receptors, which can in some cases be destroyed by different proteases, appear to show limited cross reactivity: for example, poliovirus types 1 and 2 compete with each other for HeLa cell receptors, but type 2 poliovirus does not affect the attachment of coxsackie B3 virus to this cell (Crowell, R.L., 1966). The nature of the virus-receptor interaction appears to be exceedingly complex and has not yet been fully analyzed. In 1971, Crowell and Philipson reported that coxsackie B3 virions which had been attached to HeLa cells and subsequently eluted were no longer infectious since they were unable to reattach. Loss of infectivity in this case and in analogous studies with poliovirus (Breindl, 1971) was correlated to the loss of VP·4 (δ), which suggested that the smallest capsid polypeptide was the "attachment protein". Recent evidence by Butterworth et al (1975) has disproved this theory and provided convincing support for an alternate hypothesis, namely, that the conformational integrity of the entire virus particle determines the recognition unit. These authors have shown that preparations of rhinovirus may be resolved into two populations by isoelectric focussing. Although both groups have the same polypeptide composition (including VP·4) those particles whose low isoelectric point suggests an altered conformation of capsid polypeptides do not attach to susceptible cells.

Attachment appears to pass through several stages from a loose to a tight bond with the cell, and the conversion may be temperature dependent (Lonberg-Holm and Philipson, 1974). A loose attachment is

defined as one from which infectious virus can be recovered by washing with solutions of physiological pH and ionic strength, while a tight bond is one that can be broken only by exposure to low concentrations of detergents. Lonberg-Holm and Philipson (1974) have presented a model in which an initial weak and readily reversible interaction between virus and receptor is followed by the diffusion of more receptors in the plane of the membrane to the initial site of interaction resulting in a strong multivalent attachment. Lowering the temperature would decrease the fluidity of the membrane lipids and the mobility of receptors within the membrane. According to this model the receptors for those viruses whose attachment is essentially temperature-independent such as Mengo (Mak et al, 1970), polio (Bachtold et al, 1957) and FMDV (Brown et al, 1962) must be clustered on the cell surface while the receptors for rhinovirus, whose attachment is temperature-sensitive (Lonberg-Holm and Korant, 1972), must be distributed throughout the membrane.

Uncoating and penetration of the virion

The first step in the process of uncoating picornaviruses appears to be an alteration of the structure of the capsid to facilitate the release of RNA. This conformational change may take place at the extracellular surface of the cell membrane shortly after attachment of a virion to its receptors. In the case of the enteroviruses and rhinoviruses, the product of this step in the uncoating process is stable and can be shed from the cell surface into the surrounding medium (Joklik and Darnell, 1961; Lonberg-Holm and Korant, 1972; Fenwick and

Cooper, 1962). The eluted particles, called A particles, have altered capsid morphology and antigenic properties. They contain the complete viral genome in an RNase-resistant form, but are non-infectious since they cannot re-attach to susceptible cells (Joklik and Darnell, 1961; Mandel, 1971; Lonberg-Holm and Korant, 1972; Crowell and Philipson, 1971; Breindl, 1971). This morphological rearrangement coincides with the loss of VP·4, the smallest capsid polypeptide (Crowell and Philipson, 1971; Breindl, 1971).

In the case of the cardioviruses, extracellular uncoating may be even more extensive. Hall and Rueckert (1971) have reported that a fraction of the ME virions attached to cells at 37°C undergo a conformational rearrangement which results in the release of the viral RNA into the extracellular fluid, followed by dissociation of the empty capsids in 14S pentamers.

The fact that agents such as glutathione which stabilize the native conformation of polio (Lonberg-Holm _et al_, 1975) and rhinoviruses (Lonberg-Holm and Noble-Harvey, 1973), as reflected by increased heat stability, also inhibit plaque-formation suggests that conformational rearrangement of the capsid is required for the establishment of an infection. In contrast, the presence of 40 mM $MgCl_2$ which protects ME-virus (a cardiovirus) against heat inactivation increases the efficiency of plaque formation by attached virions (Hall and Rueckert, 1971). It is most likely that the stabilizing effect of the addition of moderate amounts of salt to this system is to slow morphological alterations of the capsid and thus to reduce the number of rearrangements which would otherwise result in abortive infection through loss of the viral RNA. The increased efficiency of plaque formation by cardioviruses resulting

from slight increases in the pH of the medium in which cells and virus interact may be explicable on similar grounds (Colter et al, 1964; Hall and Rueckert, 1971).

Precisely how the virion traverses the plasma membrane is not clear. Electron microscopic evidence for a pinocytotic process is not compelling, particularly since the experiments were performed late in the infectious cycle (Dales et al, 1965). Dunnebacke et al (1969) have published electron micrographs of thin sections of cells collected at 0, 2 min, 5 min and 10 min after infection with poliovirus which seem to show direct penetration of adsorbed particles.

The instability of cardiovirus capsids suggests that RNA alone may enter the cell. The idea does not seem unreasonable since the isolated genomes of many viruses are infectious (for review, see Pagano, 1970). In addition, Chan and Black (1970) have shown that incubation of poliovirions with membranes isolated from susceptible cells renders the RNA nuclease sensitive. Alternatively, it may well be that penetration of the membrane and the conformational rearrangements of the capsid which result in release of viral RNA occur virtually simultaneously.

Alterations of cellular metabolism

Infection of cells with picornaviruses normally leads to a rapid and substantial inhibition of host DNA, RNA and protein synthesis. These effects begin very shortly after infection and are quite distinct from the progressive decay in cell functions which accompanies cell death. Elucidation of the mechanisms involved has been difficult since the rate and extent of shut-off depends on the multiplicity of infection as well as on the strain of virus and cell involved.

Inhibition of host-cell protein synthesis appears to result from dissociation of polysomes, leaving ribosomes free to associate with viral message (Penman et al, 1963). Normally, mammalian messengers have a long half-life (Greenberg, 1972). It has been established that the inhibition is not due to extensive degredation of pre-existing cellular mRNA, although it is difficult to detect very small changes in size which may be sufficient to render the message inactive (Colby et al, 1974). Leibowitz and Penman (1971) have suggested that shut-off is due to more efficient initiation of translation of viral RNA than of cellular mRNA in infected cells. This notion has recently received support from Nuss et al (1975) who have shown that translation of viral mRNA continues while initiation of translation of cellular mRNA is blocked by the addition of excess sodium chloride to the medium in which infected cells are incubated. In a preliminary communication, Thach et al (1975) have reported that while extracts of EMC-infected mouse myeloma cells translate a cellular mRNA efficiently, the translation is abolished by the addition of small amounts of viral mRNA to the system. The data indicate a competition between the two messengers for some component of the translation machinery. Specific host initiation factors are required for the translation of viral RNA in vitro (Wigle and Smith, 1973). Whatever the mechanism, it is clear that shut-off is a viral function since it does not occur if viral protein synthesis is blocked (Franklin and Baltimore, 1962; Penman and Summers, 1965). However, whether it is the direct result of the activity of a specific viral gene product either positively or negatively affecting the translation process, or whether it merely reflects the competition between viral RNA and cellular mRNAs for certain cellular factors required for

translation is less certain.

It has been suggested that double-stranded (ds) RNA, which is a byproduct of replication of the viral RNA (see next section), may be involved in the shut-off of host protein synthesis. Double-stranded RNA was shown to inhibit the initiation of protein synthesis in rabbit reticulocyte lysates *in vitro* (Ehrenfeld and Hunt, 1971), and it has been proposed that it does so by complexing with the initiation factor IF-3 (Kaempfer and Kaufman, 1973). However, Celma and Ehrenfeld (1974) found that viral and cellular protein synthesis are equally sensitive to such inhibition, and also calculated that inhibitory concentrations of ds RNA, as determined from *in vitro* studies, occur *in vivo* only late in infection, when all protein synthesis begins to decline. Although one must be cautious about extrapolating results obtained from an *in vitro* to an *in vivo* system, these observations make it seem unlikely that ds viral RNA plays a role in the shut-off phenomenon.

The inhibition of RNA synthesis by picornaviruses (Franklin and Baltimore, 1962) is also a viral function (Baltimore *et al*, 1963). Its mechanism is unknown and the possibility that the phenomenon is secondary to the shutdown of host protein synthesis has not been excluded. Thus, either lack of synthesis of some labile factor required for transcription, or the sequestering of such a factor as part of a viral replicase would have the incidental effect of blocking transcription of cellular DNA.

It has been shown that the inhibition of chromosomal DNA replication by picornaviruses can be mimicked by antibiotic-mediated inhibition of protein synthesis (Hand, 1975; Hand *et al*, 1971) and, therefore, it

is likely that this effect is secondary to the viral-mediated shut-off of protein synthesis. There is no degredation of the DNA in either case.

Replication of the Viral RNA

Studies of the kinetics of poliovirus replication show that synthesis of viral RNA begins about one half hour after infection and continues at an exponential rate until early in the logarithmic phase of viral production (about 3 hrs p.i. in this system). Thereafter, synthesis continues at a linear rate for about 1 hr and then begins to decline (Baltimore et al, 1966; Darnell et al, 1967). The switchover from exponential to linear kinetics occurs at about the time that production of progeny virions reaches its maximum rate, and it has been suggested (Balimore, 1969) that the removal of newly synthesized viral RNA for encapsidation, which limits the size of the pool of replicating molecules, is responsible for the switch. The final decline is probably due to degeneration of cellular metabolism.

The precise mechanism of replication was clarified as a result of studies on RNA phages of the f2 group (for review, see Weissman et al, 1968, 1973; Speigelman et al, 1968). The vRNA serves as template for the simultaneous transcription of six to seven strands of complementary RNA (cRNA; Baltimore, 1968). The cRNA molecules then serve as templates for the synthesis of viral RNA molecules in an analogous fashion. The transcribing structures, called replicative intermediates (RI), thus consist of a single strand of template RNA hydrogen-bonded to the growing 3' ends of progeny molecules whose completed 5' ends are free (Thach et al, 1974;

for review, see Bishop and Levintow, 1971). Synthesis of RNA is asymmetric in that most of the RI's produce viral RNA (Bishop and Levintow, 1971). Whether this is a result solely of the removal of vRNA for translation or encapsidation is not known. The precursor nature of the RI was clearly demonstrated by pulse-chase experiments (Girard, 1969; McDonnell and Levintow, 1970) in which radioactivity was shown to enter RI structures rapidly and then to pass into single stranded progeny molecules. In infected cells, the entire complex is firmly bound to smooth membranes.

The double helical "replicative form" (RF) which accumulates late in infection (Baltimore and Girard, 1966) has been shown by pulse-chase experiments to be an irrelevant end product of the degeneration of RI's (Baltimore, 1968; Girard, 1969).

Since the synthesis of RNA from RNA templates does not appear to be a normal cell function, it was assumed that no pre-existing cellular enzyme _per se_ would suffice to replicate the genomes of picornaviruses, an assumption that was strengthened by the observation that these viruses grow in the presence of Actinomycin D which inhibits DNA-dependent RNA polymerase (Reich _et al_, 1962). Shortly thereafter, a virus-induced RNA-dependent RNA polymerase (replicase) was detected in cells infected with Mengo and polio viruses (Baltimore and Franklin, 1963). The association of replication complexes with smooth membranes, together with the marked instability of the replicase activity has severely hampered efforts to carry out definitive studies of the enzyme. The membrane-bound complex as a whole may be isolated in active form, but dissociation from membranes usually results in a rapid, irreversible loss of replicase activity (Girard _et al_, 1967).

Some progress has been made. Ehrenfeld et al (1970) have succeeded in purifying the replicase complex 85-fold without loss of activity. SDS-polyacrylamide gel analysis of a purified complex from EMC virus-infected cells has revealed the presence of 5 polypeptides (MW = 72,000, 65,000, 57,000, 45,000 and 35,000) one of which has a molecular weight (57,000) identical to that of the viral non-structural polypeptide E (Rosenberg et al, 1972). The molecular weights of the others are remarkedly similar to those of the four subunits of Qβ replicase: 70,000, 65,000, 45,000 and 35,000; subunit III, (MW = 45,000) is virus coded in this system (Kondo et al, 1970; Kamen, 1970). A similar analysis of the replicase extracted from radiolabelled poliovirus-infected cells showed that it also contains a virus-specific polypeptide which co-migrates with the poliovirus equivalent of polypeptide E (Lundquist et al, 1974). These observations suggest that the replication of picornaviral RNAs, like those of some RNA phages, may be catalyzed by an enzyme composed of a viral polypeptide (polypeptide E in the case of the cardioviruses) in association with pre-existing, and as yet unidentified, cellular polypeptides.

Cooper et al (1970) have presented genetic evidence for the existence of two virus-specific polymerase activities. However, due to the monocistronic nature of the genome (see next section) the existence of two separately mutable sites cannot be taken as conclusive evidence for the existence of two separate polypeptides.

Biosynthesis of Viral Polypeptides[1]

The synthesis of picornaviral polypeptides takes place on large

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[1]For review, see Hershko and Fry, 1975.

(380 S) polysomes in which the entire viral RNA serves as messenger (Penman et al, 1964). From the kinetics of labelling of the 14 or so virus-specific polypeptides found in infected cells, and from a consideration of their combined molecular weights together with the coding capacity of the viral genome, it was concluded that stable virus-specific proteins are formed by post-translational cleavage of larger precursors (Summers and Maizel, 1968; Maizel and Summers, 1968; Holland and Kiehn, 1968; Jacobson and Baltimore, 1968). Jacobson and Baltimore (1968) suggested that the viral RNA is translated into a single giant polypeptide ('polyprotein') from which all viral proteins are produced by subsequent cleavages.

In spite of the fact that polyprotein is not seen under normal conditions, considerable evidence has accumulated to support this hypothesis. A giant polypeptide, MW > 200,000, has been detected in lysates of coxsackie virus-infected cells after a very short pulse label early in infection (Kiehn and Holland, 1970). Polyprotein has also been detected in infected cells in which proteolytic cleavage was blocked by specific inhibitors such as TPCK or TLCK (Korant, 1972; Summers et al, 1972), by zinc ions (Butterworth and Korant, 1974), or by the incorporation of amino acid analogues into the primary sequence of the protein (Jacobson et al, 1970; Paucha et al, 1974). Some temperature-sensitive mutants of poliovirus have been shown to accumulate high molecular weight precursors which are cleaved when the temperature is restored to the permissive level (Garfinkle and Tershak, 1971). Roumiantzeff et al (1971) reported that membrane-bound polyribosomes from poliovirus-infected cells synthesized a protein in vitro

whose size suggested that it was produced by uninterrupted translation of the viral genome. Also compatible with the hypothesis are the results of studies of picornaviral RNA-directed protein synthesis in cell free systems, which show that (at least in vitro) these molecules contain a single site (at or near the 5' end) for the initiation of translation (Öberg and Shatkin, 1972; Boime and Leder, 1972; Smith, 1973).

The cleavage steps which result in the formation of picornavirus polypeptides are presented in Fig. 1. This scheme, first proposed by Butterworth and Rueckert (1972a), was deduced from pulse-chase experiments using EMC virus-infected cells, and verified by genetic mapping of the polypeptides (1972b) using the drug pactamycin which at low concentration inhibits initiation of translation (Summers and Maizel, 1971; Taber et al, 1971; Rekosh, 1972), and by cyanogen bromide and tryptic mapping of isolated polypeptides (Butterworth et al, 1971; Dobos and Plourde, 1973). Pactamycin mapping and kinetic studies have shown that the pattern of cleavage is very similar for poliovirus (Butterworth, 1973), rhinovirus 1A (Butterworth, 1973; McLean and Rueckert, 1973) and for Mengovirus (Paucha et al, 1974; Lucas-Lenard, 1974). As this scheme will be discussed fully in Chapter III, only a brief resumé is presented here.

Under normal conditions, three large polypeptides (designated A, F and C) are produced by primary cleavages of the nascent 'polyprotein'. Protein A, the precursor of the four capsid polypeptides is located at the amino-terminal end of the polyprotein (corresponding to 5' region of the viral RNA), and product C is located at the carboxy-terminal

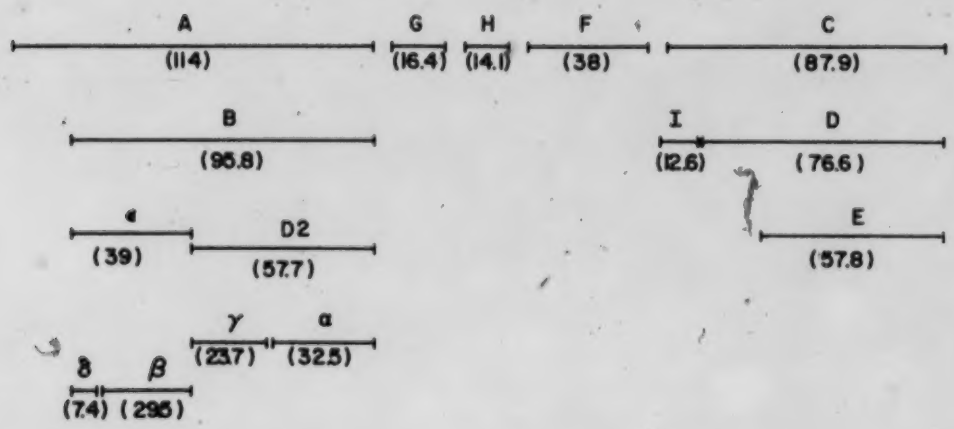

Figure 1. Cleavage scheme of Mengo virus-specific polypeptides. The numbers in brackets refer to the molecular weight, in thousands, of each polypeptide.

end (corresponding to the 3' region of the viral genome). The cleavages, C → D → E and A → ε, α and γ, have been termed secondary cleavages. The morphogenetic cleavage, ε → δ + β, occurs during the final stages of virion assembly. Polypeptide F is stable.

The nature of the enzyme or enzymes involved in this process is unknown. The observation that the inhibitors TPCK and TLCK block the primary cleavages of the polyprotein (Korant, 1972; Summers et al, 1972) suggests that they may be catalyzed by an enzyme having chymotrypsin-like activity. It is also highly likely that these cleavages are catalyzed by a cellular protease or proteases, since Korant (1972) has shown that the poliovirus polyprotein, isolated from monkey kidney cells infected in the presence of TPCK, can be converted, by incubation with extracts of uninfected cells, into products identical to those produced by primary cleavages in vivo. Secondary cleavages were found to occur only in the presence of extracts of infected cells, which suggests that a viral protease is involved at this stage.

From an analysis of the termini of isolated Mengo virion polypeptides, Ziola and Scraba (Virology, in press) have suggested that the secondary cleavages resulting in the formation of ε, α and γ are catalyzed by a single viral protease with a specificity for peptide bonds involving the carbonyl function of glutamine.

Lawrence and Thach (1975) have examined the synthesis and cleavage of the capsid precursor in vitro and have found that an early stage in its processing involves the removal of a small fragment from the amino-terminal end. The proteolytic activity responsible for this cleavage appears to co-purify with capsid protein γ. Since the γ sequence is

an integral part of A, these authors have suggested that the process may be autocatalytic. This same activity may also be responsible for the final morphogenetic cleavage, since Korant (1973) has shown that the conversion of $\varepsilon \rightarrow \delta + \beta$ does not take place in the absence of capsid assembly.

Virion Assembly[1]

During the replication of poliovirus in HeLa cells, empty capsids (sed.coeff. = 73S), whose polypeptide composition differs from that of mature virions in that they contain VP·0, the precursor of VP·2 and VP·4, in uncleaved form (Maizel *et al*, 1967), are produced. Jacobson and Baltimore (1968b) found that 73S empty capsids accumulate in infected cells in the presence of 3 mM guanidine hydrochloride, and since the radioactively-labelled protein therein was found to shift into mature virions upon removal of the guanidine, they concluded that the 73S particle is a precursor in the assembly process. Fernandez-Tomas and Baltimore (1973) subsequently detected a 125S particle which contained viral RNA, but in which VP·0 was also uncleaved, suggesting that the "morphogenetic" cleavage of VP·0 → VP·2 + VP·4 is not essential for insertion of the viral RNA into the preformed capsid but may be required for stabilization of the capsid structure.

Phillips *et al* (1968) had previously shown that poliovirus-infected HeLa cells contain 5S and 14S structures with the same polypeptide composition as empty capsids. The 14S structures could be assembled *in vitro* into 73S procapsids (Phillips, 1969, 1971). The

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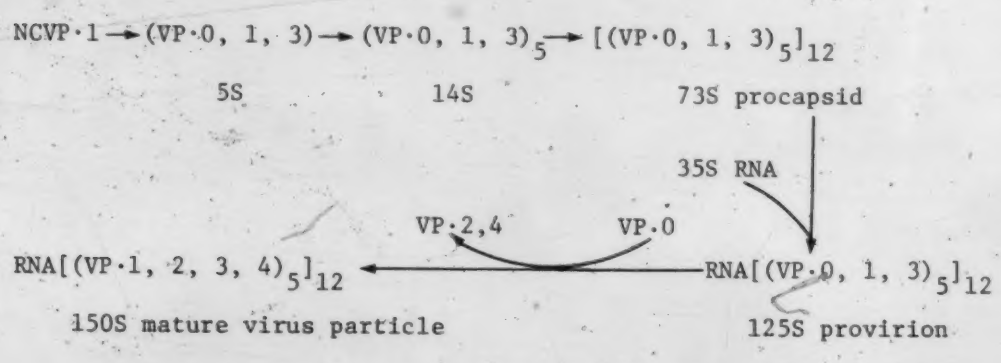
[1]For reviews, see Casjens and King, 1975 and Hershko and Fry, 1975.

reaction was found to be enhanced by the addition of rough membranes from infected cells (Perlin and Phillips, 1973), suggesting that the self-assembly of 14S precursors *in vivo* occurs on rough cytoplasmic membranes which have been modified during infection.

On the basis of this information, the following scheme has been suggested for the assembly of poliovirus (Casjens and King, 1975; Phillips, 1972)



A major criticism of the experiments on which this assembly scheme is based is that it is difficult to define product-precursor relationships unambiguously on the basis of pulse-chase experiments due to the large pool sizes of the intermediates. It is possible that some species are the products of abortive assembly rather than structural intermediates. Most suspect in this regard is the 73S procapsid. Ghendon *et al* (1972) have found that poliovirus-infected MiO cells (from rhesus monkey tonsils) accumulate 14S rather than 73S particles in the presence of guanidine. When the guanidine is removed, the 14S particles chase directly into mature virions without the appearance of 73 procapsids. *In vitro*, however, empty capsids do form.

Although empty capsids are regularly found during infection with poliovirus, they are never found in cells infected with the cardioviruses. McGregor *et al* (1975) have found two capsid precursors in EMC

virus-infected HeLa cells which sediment at 13S and 14S in glycerol gradients. The 13S particle, of polypeptide composition $(A)_5$, was found to be converted into the 14S particle of polypeptide composition $(\varepsilon,\alpha,\gamma)_5$. Since $(\varepsilon,\alpha,\gamma)$ was never found in monomeric form, these workers have suggested that polypeptide A must aggregate into a pentamer before undergoing secondary cleavages. They have proposed that the assembly of cardioviruses proceeds according to the following scheme.

$$\underset{13S}{(A) \rightarrow (A)_5} \rightarrow \underset{14S}{(\varepsilon\alpha\gamma)_5} \xrightarrow[\varepsilon \quad \delta + \beta]{RNA} \underset{150S\ virion}{RNA\cdot[(\delta\beta\gamma\alpha)_{58-59}\ (\varepsilon,\alpha,\gamma)_{1-2}]}$$

It would appear from these data that the assembly of cardioviruses occurs in a highly concerted fashion. Prather and Taylor (1975) have reported that 80S and 125S subviral particles are produced during the replication of Mengovirus in a restrictive bovine kidney cell line (MDBK). However, no attempt was made to characterize these particles.

While further work will be required to confirm and define more precisely the observed differences between the assembly pathways of entero- and cardioviruses, it seems likely that they reflect real and fundamental differences in the nature of the intermolecular bonding between the capsid polypeptides of different picornaviral subgroups.

The work described in this thesis was undertaken to identify the virus-specific polypeptides produced in Mengo virus-infected L.cells and to define the post-translational cleavage scheme by which they are produced. One objective of these studies was to determine whether any

differences in the production of polypeptides by the L-, M- and S-variants of Mengo virus (Ellem and Colter, 1961) could be detected. The results of comparable studies of the poliovirus-HeLa cell system are presented, as are preliminary data obtained from attempts to establish a cell-free protein synthesizing system from Mengo virus-infected L.cells. Finally, the identification and partial characterization of a subviral particle, believed to be an intermediate in the assembly of Mengo virus, is described.

II. ROUTINE MATERIALS AND METHODS

Media

Sterilization procedures

All media were sterilized by filtration through a nitrocellulose filter (Millipore Corporation, Bedford, Ma.) with a pore size of 0.22μ (microns).

Growth Media

Eagle's minimum essential medium for suspension cultures containing spinner salts and glutamine (catalogue number F-14) and Eagle's basal medium (BME diploid) with Earle's salts and glutamine (catalogue number G-13) were obtained in powder form from the Grand Island Biological Company, Grand Island, New York. The media were dissolved in distilled, deionized water and sodium bicarbonate was added to a final concentration of 0.12% before filtration.

Before use these media were supplemented with:

(1) Horse serum (Flow Laboratories, Rockville, Md.) to a final concentration of 5% for growth of cells or 1% for production of virus,

(2) Penicillin G (Glaxco-Allenburys Ltd., Toronto, Ont.) and streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) to final concentrations of 100 IU and 50 μg/ml respectively.

Amino acid deficient medium

This medium was similar in composition to Eagle's basal medium (diploid) except that it contained twice the normal amount of calcium chloride (i.e. 400 mg/l) and no amino acids other than glutamine. Sodium bicarbonate (final conc. = 0.06%) was added before filtration,

after which the sterile medium was supplemented with 1% horse serum and antibiotics as described above.

Virus diluent

The phosphate-buffered saline (PBS) of Dulbecco and Vogt (1954) was supplemented with 0.2% bovine serum albumin, fraction V (Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.), 0.002% phenol red (J.T. Baker Chemical Co., Phillipsburg, N.J.) and twice the usual concentration of antibiotics.

Overlay diluent

This solution contained three times the normal concentration of Hanks' salts, six times normal concentrations of both Basal Eagle's amino acids (Baltimore Biological Laboratory, Division of Becton Dickinson and Company, Cockeysville, Md.) and MEM vitamin solution (Gibco), five times the usual concentrations of penicillin and streptomycin, 0.78% sodium bicarbonate and 30% inactivated (56°C for 45 min) calf serum.

Agar overlay

Prepared by mixing 1 volume of overlay diluent with two volumes of a 1.6% distilled water solution of Noble agar (Difco Laboratories, Detroit, Mich.) at 45°C.

Cultured L. Cells

Earle's L-929 strain of mouse fibroblasts (Sanford *et al*, 1948) were used for the propagation and assay of Mengo virus. They were obtained originally from the American Type Culture Collection, Rockville, Md.

Growth of cells

L. cells were maintained in monolayer culture in 1-litre Blake bottles (Kimble Products, Owens-Illinois Co., Toledo, Ohio). When the cultures had reached confluence the growth medium was removed and the monolayers rinsed with a solution of 0.25% trypsin (Difco) in a buffer containing 10 mM phosphate pH 7.4, 142.8 mM sodium chloride and 2.8 mM potassium chloride and incubated at 37° until the cells began to stream off the glass (1 to 2 min). Cells from several bottles were resuspended in BME-5% H.S. and used to maintain the Blake bottle stock, while the remaining cells were resuspended in spinner medium and transferred to 1- or 2-litre spinner flasks (Bellco Biological Glassware, Vineland, N.J.) at a concentration of 2×10^5 cells/ml. The cells were kept in suspension by means of a magnetic stirring device while growing at 37°C.

Virus

The large-, medium- and small-plaque variants of Mengo encephalomyelitis virus, originally isolated by Ellem and Colter (1961), were used throughout these studies.

Virus Growth in Roller Bottles

Confluent L. cell monolayers were grown in large cylindrical bottles (490 mm x 110 mm diameter - Bellco Biological Glassware) coated with fetal calf serum (Flow Laboratories) prior to seeding to facilitate the attachment of cells. The L. cells were harvested from spinner culture, resuspended in fresh growth medium at a density of about 10^6 cells/ml and dispensed in 150 ml aliquots to the coated roller bottles. The bottles were rotated on a Bellco roller apparatus at 0.2 rpm for

3 hr after which the speed was increased to 1 rpm. Monolayers reached confluence after about 48 hr of growth at 37°C. The growth medium (containing 5% horse serum) was then replaced by 20 ml of growth medium (with 1% horse serum) which contained the virus inoculum (about 10^8 pfu/ml corresponding to a multiplicity of about 10). The bottles were then rotated at 0.2 rpm for 3 hr and at 1 rpm for an additional 20 to 24 hr. At the end of this time, most of the cells had lysed; any remaining cells were dislodged from the glass by shaking.

Virus Purification

The procedure used for virus purification, a modification of that developed by Scraba et al (1967), has been described by Ziola and Scraba (1975).

Lysates of infected L. cells were pooled and centrifuged at 1,000g for 15 min. The pellet was resuspended in a small volume of distilled water, frozen and thawed three times to release trapped virus and recentrifuged. The combined supernatants were chilled in ice, after which the virus was precipitated by the addition of cold (-15°) methanol to a final concentration of 20% and allowing the solution to stand overnight at -15°C. The precipitate was collected by centrifugation at 5000g for 30 min, suspended in 0.2 M sodium phosphate buffer, pH 7.8, and homogenized by hand in a Potter-Elvehjem tissue homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.) to disrupt large aggregates. A solution of α-chymotrypsin (3x recrystallized-Worthington Biochemicals, Freehold, N.J.) was added to give a final enzyme concentration of 0.8 mg/ml and the mixture incubated for 20 min at 37° with stirring. An equal volume of 0.2M sodium pyrophosphate, pH 8.0, was added and

incubation continued for an additional 20 min. The mixture was then chilled and clarified by centrifugation at 20,000g for 10 min. Virus was pelleted from the supernatant by centrifugation at 120,000g for 60 min, and resuspended by homogenization in a small volume of 0.1M sodium phosphate buffer, pH 7.4.

The partially purified virus was sedimented through a discontinuous gradient made of equal (13 ml) volumes of 15% and 30% sucrose by centrifugation for 20 hr at 20,000 rpm in a Spinco SW27 rotor. The pellet was again resuspended by homogenization in 0.1M sodium phosphate, pH 7.4, and mixed with an aqueous solution of Cs_2SO_4 to a final density of 1.31 g/cc. After centrifugation for 20 hr at 40,000 rpm in a Spinco SW50.1 rotor, the virus, which appeared as a white band near the middle of the gradient, was collected through the side of the tube with a syringe. Salt was removed by passage through a 3 cm^2 x 20 cm column of Sephadex G25 and the virus was stored at 4^oC in 0.1M sodium phosphate buffer, pH 7.4.

Preparation of Radioactivity Labelled Virus

Confluent monolayers of L. cells in roller bottles were infected with Mengo virus in the amino acid deficient medium described. Ninety minutes to 2 hr after infection, ^3H- or ^{14}C-labelled amino acid mixtures (New England Nuclear, Montreal, Que., catalogue numbers NET-250 and NEC-445 respectively. Both mixtures contain defined amounts of 15 highly purified amino acids.) were added to give final concentrations of 2 μCi/ml or 0.2 μCi/ml respectively. Subsequent procedures were identical to those already described.

Plaque assay of infectious virus

The procedure has been described by Campbell and Colter, 1965.

Monolayers of L. cells on 60 x 15 mm plastic petri dishes (Falcon Plastics, Oxnard, Ca.) were prepared by harvesting cells from Spinner culture, resuspending at a density of 5×10^5 cells/ml in fresh growth medium, and adding 5 ml of this suspension to each plate. Confluence was reached after incubation for 24 hr at 37° in a humidified atmosphere of 5% CO_2 in air.

The medium was aspirated and each plate innoculated with 0.1 ml of appropriate dilutions of virus in virus diluent. After incubation for 1 hr at 37^oC to allow virus to attach, each plate was overlaid with 4.5 ml of agar overlay.

Plaques were visible after incubation at 37° for an additional 48 hr. In order to facilitate counting, the plates were stained at this time by addition of 3 ml of agar overlay containing 0.01% neutral red (Fischer Scientific, Fair Lawn, N.J.).

CHAPTER III: BIOSYNTHESIS OF MENGOVIRUS-SPECIFIC POLYPEPTIDES

Introduction

At the time that this work was undertaken, it had been shown in a qualitative way that the stable, virus-specific polypeptides synthesized during the replication of the enteroviruses polio (Jacobson and Baltimore, 1968a; Summers and Maizel, 1968; Maizel and Summers, 1968; Holland Kiehn, 1968) and coxsackie (Kiehn and Holland, 1970), and of the cardioviruses Mengo (Kiehn and Holland, 1970) and EMC (Butterworth et al, 1971) are produced by post-translational cleavage of large precursor polypeptides. In addition, Butterworth et al (1971) had proposed a cleavage scheme for the formation of EMC virus-specific polypeptides.

The results of experiments described in this chapter (i) confirm that the virus-specific polypeptides synthesized in Mengo virus-infected L. cells are produced by a process of post-translational cleavage of large precursor molecules, (ii) establish the gene order of the primary and of the stable viral gene products, (iii) show that the pattern of post-translational cleavages involved is very similar to that proposed for the production of EMC virus-specific polypeptides and to those subsequently described for the poliovirus (Butterworth, 1973) and rhinovirus 1A (McLean and Rueckert, 1973) systems, and (iv) suggest that the synthesis of Mengo virus-specific polypeptides is subject to some kind of translational control.

Materials and Methods

Cells and virus

Cells of Earle's L-929 strain of mouse fibroblasts were grown in suspension culture in calcium-free Eagle's minimum essential medium (Gibco) supplemented with 5% horse serum. Confluent monolayer cultures were prepared by dispensing aliquots of 2.5×10^6 cells in 5 ml Eagle's basal minimum essential medium containing 5% horse serum (BME + 5% HS) into 60 mm x 15 mm plastic petri dishes (Falcon) 24 hr before use.

The three plaque variants (L, M and S) of Mengo virus used in these studies were those isolated originally by Ellem and Colter (1961). Virus was purified as described in Chapter II.

Infection of monolayers and preparation of cell lysates

Confluent monolayers (approximately 5×10^6 cells) were infected at an estimated multiplicity of 100 pfu/cell with Mengo virus suspended in 0.2 ml of virus diluent containing 5 μg Actinomycin D/ml (Schwarz/Mann Biochemicals, Orangeburg, N.Y.). After incubation for 1 hr at 37° to permit attachment of the virus, the monolayers were washed once with warm (37°) BME + 5% HS, and then incubated in 5 ml of the same medium containing 5 μg actinomycin D/ml (C-medium). At 5 hr post-infection, the C-medium was replaced by 5 ml of amino acid-deficient Eagle's medium containing 1% horse serum, 5 μg actinomycin D/ml, and 15 mM N-2-hydroxyethylpiperazine - N'-2-ethanesulfonic acid (HEPES, Sigma) pH 7.0 (D-medium), and incubation was continued for one hour in order to deplete the intracellular amino acid pools. At 6 hr post-infection, cells were pulse labelled for 15 min by removing the D-medium and adding 2 ml of warm (37°) D-medium containing 50 μCi/ml ^{3}H-amino acids

(New England Nuclear, NET-250). In those experiments in which labelling was followed by chase period, the monolayers - after removal of the radioactive medium - were washed once with and then incubated for the desired length of time in C-medium.

Infection and labelling in the presence of amino acid analogues was carried out as described above with two modifications. Ten minutes before the addition of the radioactive amino acids, the D-medium was replaced by 2 ml of warm (37°) D-medium containing 3.1 mM p-fluorophenylalanine (Sigma), 3.6 mM canavanine (Nutritional Biochemicals), 8.0 mM azetidine-2-carboxylic acid (Aldrich Chemical Co., Milwaukee, Wis.) and 2.2 mM ethionine (Sigma). Cells were then labelled for 30 min (6.0 to 6.5 hr post-infection) by incubation in the same medium supplemented with ^{3}H-amino acids (50 μCi/ml).

In all cases, lysates were prepared by removing the medium, washing the monolayers three times with 3 ml volumes of cold PBS and adding 0.2 ml of "lysis mixture": 0.01 M sodium phosphate buffer, pH 7.2, containing 2% sodium dodecyl sulfate (Matheson, Coleman and Bell, Norwood, Ohio), 5% β-mercaptoethanol (Baker) and 10^{-3} M phenylmethylsulfonylfluoride (PMSF, Sigma). Lysates were stored at -20° until analyzed.

Immune Precipitation of Mengo-Specific Polypeptides

Antiserum against the capsid polypeptides of Mengovirus (M-variant) was produced in rabbits using heat-disrupted virions as antigen. One mg of viral protein was injected intravenously followed 14 days later by an intramuscular injection of the same amount of protein suspended in Freund's adjuvant. Twenty-one days after the intramuscular injection,

the animals received a second intravenous injection. Blood was collected one week after the last injection, and the γ-globulin fraction was separated from the serum by precipitation with ammonium sulfate (Schwarz-Mann).

Lysates for immune precipitation were prepared by adding 0.5 ml PBS containing 0.5% Nonidet P.40 (NP.40, Shell Oil of Canada) to the washed monolayers. After removal of nuclei by a brief centrifugation (10 min, 1000g) 5 to 25 μl aliquots of the solution of immune γ-globulin were added to the clarified lysates. The mixtures were incubated for 30 min at 37°C at which time equal volumes of goat anti-rabbit γ-globulin (Miles Laboratories, Kankakee, Ill.) were added and incubation continued for an additional 2 hr .

The immune precipitates were collected by centrifugation, washed twice with PBS containing 0.5% NP.40, then dissolved in 100 μl volumes of "lysis mixture" (see preceding section) and stored at -20°C until analyzed. The supernatants from the immune precipitation were also stored at -20°. They were dialyzed for several hours against 2% SDS-5% β mercaptoethanol - 0.01 M sodium phosphate (pH 7.4) before being analyzed by SDS-polyacrylamide gel electrophoresis.

Virus growth

In order to verify that virus replication had proceeded normally, single cycle growth was examined in each experiment. Replicate plates were infected and manipulated in the manner described above except that they were not pulse labelled. At hourly intervals, from 4 to 8 hr post-infection, cells were collected from duplicate plates, disrupted by sonic vibration, and the amount of infectious virus in each clarified

lysate determined by standard plaque assay on L-cell monolayers.

Polyacrylamide gel electrophoresis

Gels, 20-21 cm in length, were prepared according to the procedure described by Weber and Osborn (1969), in 0.1 M sodium phosphate (pH 7.2) - 0.1% SDS from a stock solution of 30% acrylamide - 0.8% bisacrylamide (Eastman Organic Chemicals, Rochester, N.Y.), both recrystallized by the method of Loening (1967). Polymerization, in 6 mm (inside diameter) x 25 cm glass tubes that had been acid washed and coated with dichlorodimethylsilane (Fluka, Switzerland) was catalyzed by ammonium persulfate (50 mg/ml, Mallinckrodt Chemical Works, St. Louis, Mo.) and 0.06% N,N,N',N'-tetramethylethylenediamine (Eastman). The gels were preelectrophoresed for at least 1 hr at 8 mA/gel before the samples were applied.

Samples (50-120 µl) were prepared by mixing aliquots of ^{3}H-labelled cell lysates with ^{14}C-labelled Mengo virions to provide internal marker proteins. All samples contained 0.01 M sodium phosphate (pH 7.2), 2% SDS, 5% β-mercaptoethanol 10^{-3} M PMSF, 10% glycerol and 0.002% bromphenol blue (both from Fischer), and were immersed in boiling water for 3 min immediately before being layered onto the gels. Mengo virions are dissociated into their component polypeptides by this treatment (Ziola and Scraba, 1974). Electrophoresis was carried out at 4 mA/gel until each sample had entered the gel matrix, after which the current was increased to and held at 8 mA/gel until the bromphenol blue had migrated to within 2 cm of the end of the gel (12 to 18 hr, depending on the concentration of acrylamide used).

Gels were stained with coomassie brilliant blue (Sigma) to visual-

ize the protein bands (Weber and Osborn, 1969), after which they were fractionated manually into 1 mm slices using a brass template. The slices were transferred to glass scintillation vials, incubated overnight at 45° with 0.3 ml aliquots of a 5.3% solution of H_2O in NCS (Amersham-Searle, Oakville, Ont.) after which 5 ml of toluene scintillation fluid were added to each vial and the radioactivity therein was measured in a liquid scintillation spectrometer (Beckman Model LS-230).

Molecular weight determinations

The molecular weights of the virus-specific polypeptides were determined from their rates of migration in SDS-polyacrylamide gels relative to those of standard, marker proteins according to the method described by Shapiro *et al* (1967). The marker proteins used in these studies, together with (in brackets) their molecular weights in thousands, were as follows: *E. coli* RNA polymerase (β, β', σ and α subunits = 157, 150, 90 and 40 respectively), β-galactosidase (130), *E. coli* DNA polymerase (110), phosphorylase b (100), bovine serum albumin (68), fumarase (49), lactic dehydrogenase (36), α-chymotrypsinogen (25.7), myoglobin (17.2) and lysozyme (14.5). The polymerase and the phosphorylase b were gifts from Drs. V. Paetkau and N.B. Madsen of this department, myoglobin was purchased from Mann Research Laboratories, Orangeburg, N.Y., lysozyme from Worthington Biochemical Corp., and the remaining proteins from Sigma Chemical Company.

Pactamycin was a gift from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, N.I.H.

Results

In the experiments described below infected or mock-infected cells were pulse labelled with a mixture of ^{3}H-labelled amino acids at 6 hr post-infection. From Fig. 2, a single-cycle growth curve of Mengo virus in L. cell monolayers, it may be seen that this time corresponds to the mid to late logarithmic phase of virus production.

Comparison of L-, M- and S-Mengo

An early objective of these investigations was to determine whether any quantitative or qualitative differences existed among the three plaque variants of Mengo virus with respect to the synthesis of virus-specific polypeptides. Repeated analyses were carried out on lysates of L. cells infected with the three variants. The lysates were prepared either immediately after a labelling period (pulse) or after a labelling period followed by incubation in the absence of labelled amino acids (pulse-chase). In neither case were any differences observed; the electropherograms obtained with the three variants were reproducibly identical (data not shown). Having established this fact, subsequent studies were carried out with the M-variant, since it is somewhat more convenient to grow and assay than are the other two. The data summarized in Tables 2, 3 and 5 were pooled from experiments in which all three variants were employed.

Synthesis of viral-specific polypeptides

Illustrative SDS-polyacrylamide gel electropherograms are presented in Fig. 3. Panel A shows the characteristic electrophoretic pattern obtained with lysates of mock infected cells. By 6 hr post-infection, cellular protein synthesis is depressed so that the virus-specific

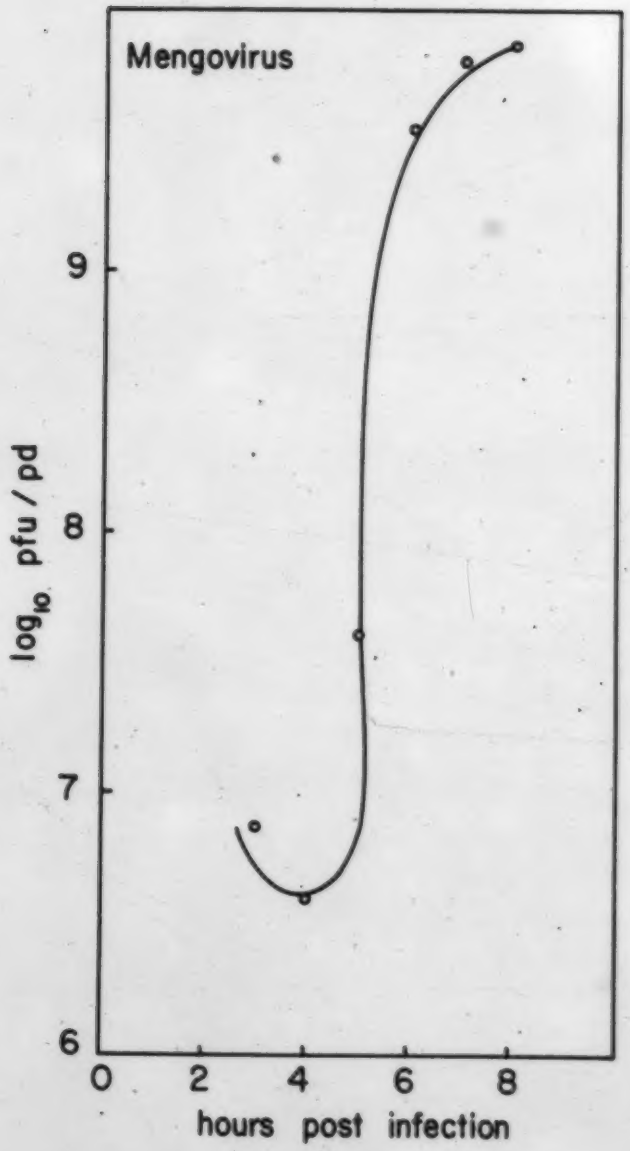


Figure 2. Single cycle growth curve of Mengo virus in L.cell monolayers. Output is expressed as pfu/60 x 15mm petri dish (i.e./≈5 x 10^6 cells).

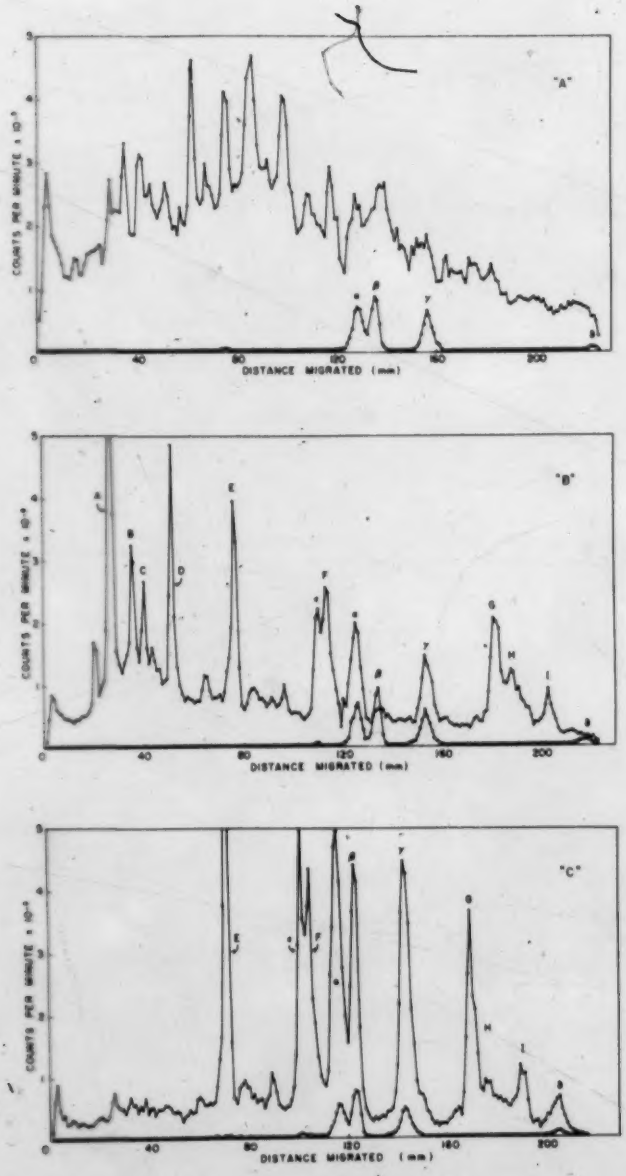


Figure 3. Profiles obtained from electrophoresis of lysates of L.cells in SDS-7.5% polyacrylamide gels. Purified ^{14}C-amino acid labelled Mengo virions were added to all samples prior to electrophoresis and the positions of the structural polypeptides derived therefrom are shown by the closed circles. All samples were pulse-labelled with ^{3}H-amino acids for 15 min at 6 hr p.i. Panel A: Mock-infected cells. Panel B: Mengo virus-infected cells. Panel C: Mengo virus-infected cells, incubated in the absence of labelled amino acids for 105 min after the pulse.

polypeptides may be readily identified. This is clear from panel B, which is an electropherogram obtained from a lysate of infected cells prepared immediately after a 15 min labelling period, and in which 12 virus-specific polypeptides are resolved (the structural polypeptides β and δ are not present in significant amounts after a 15 min pulse label). The electrophoretic pattern obtained with a lysate of cells, pulse labelled for 15 min at 6 hr post-infection and then incubated for an additional 105 min in the absence of labelled amino acids, is shown in panel C. The disappearance of radioactivity from the large precursor polypeptides A, B, C, D, and the concomitant increase of radioactivity in 10 polypeptides of lower molecular weight (which, with the exception of polypeptide ε, are stable end-products) makes it clear that with Mengo, as with all other picornaviruses studied, the capsid and non-capsid virus proteins are produced by proteolytic cleavage of one or more large precursor proteins. All unlettered peaks in Figs. 3B and 3C are believed to result from residual host protein synthesis, since they correspond to peaks present in electropherograms of lysates of mock-infected cells, and appear to be stable during a chase period of 105 min.

The molecular weights of the 14 virus-specific polypeptides identified in Fig. 3 were determined on the basis of their electrophoretic mobilities (in SDS-7.5% polyacrylamide gels) relative to those of marker proteins of known molecular weights (Shapiro et al, 1967). A representative standard curve showing the relative positions of the viral polypeptides is presented in Fig. 4. The molecular weights of those polypeptides larger than 70,000 were verified on 5% acrylamide gels such as those shown in Fig. 11. The nomenclature used is that

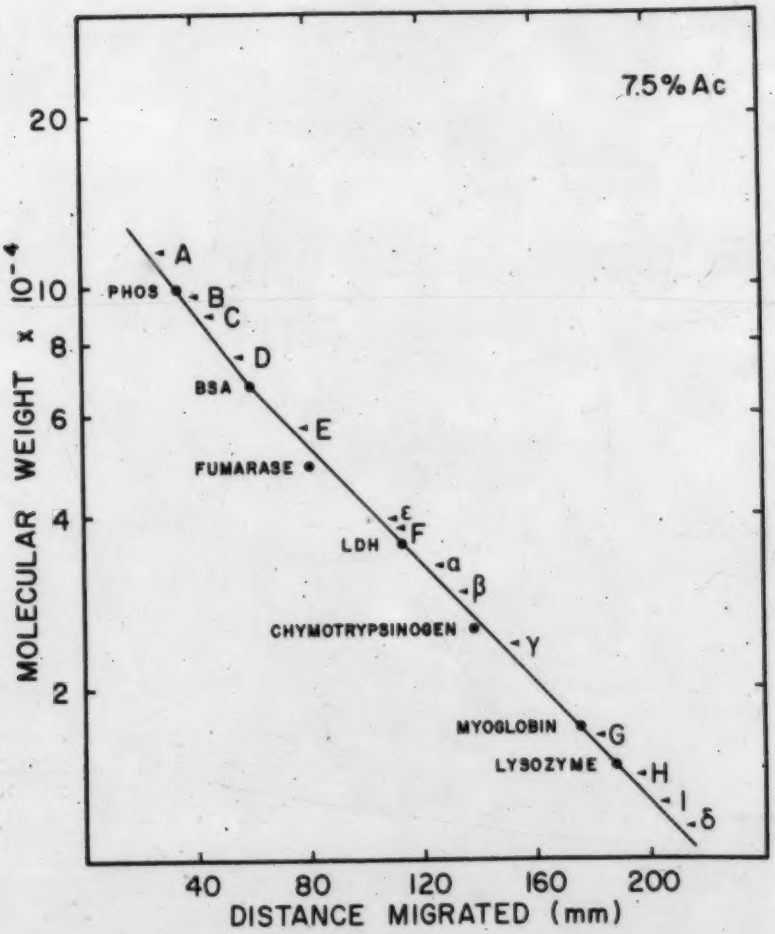


Figure 4. Molecular weights of Mengo virus-specific polypeptides as determined by migration in SDS-7.5% polyacrylamide gels. The molecular weights assigned to the marker polypeptides are listed on page . (●), position of marker polypeptide; (◄) position of viral polypeptide.

employed by Butterworth et al (1971) in their studies with EMC virus. The results are summarized in Table 2, which also indicates the kinetic behavior of each polypeptide. It should be pointed out that although polypeptide ε is classified here as an unstable precursor protein, it does occur in Mengo virus to the extent of 1-2 molecules/virion (Ziola and Scraba, 1974). Polypeptide D2, which has been shown to have a molecular weight of 57,700 and like ε, to be present to the extent of 1-2 molecules in every mature Mengo virion (Ziola and Scraba, 1974) is not listed here since it is not resolved from non-capsid protein E (MW = 57,300) in these gels. The molecular weight of the capsid polypeptide δ determined by this method is too high. Based upon amino acid composition analysis of the purified protein Ziola and Scraba (1975) have obtained a molecular weight of 7,350 for polypeptide δ.

Pactamycin mapping of the Mengo virus genome

The sequence in which Mengo-specific polypeptides are synthesized was determined from experiments in which the antibiotic pactamycin was used to block initiation of translation (MacDonald and Goldberg, 1970; Taber et al, 1971; Summers and Maizel, 1971). Inhibiting the initiation of translation of a monocistronic mRNA without affecting the elongation process results in a gradual loss of ribosomes from the message beginning with the region closest to the initiation site, the 5' end, and progressing toward the 3' terminus. As a result, when pactamycin and radioactive amino acids are given simultaneously, lower amounts of radioactivity are incorporated into the NH_2-terminal portions of the polypeptides as compared to incorporation under steady state conditions

TABLE 2

Molecular Weights and Kinetic Behavior of Mengo Virus-Specific Polypeptides

Polypeptide	Molecular weight[1]	Kinetic behavior[2]
A	114.0 ± 4.0	u
B	95.8 ± 1.1	u
C	87.9 ± 2.6	u
D	76.6 ± 1.9	u
E	57.3 ± 1.9	s
ε	39.0 ± 0.3	u
F	38.0 ± 0.5	s
α	32.5 ± 0.3	s
β	29.5 ± 0.4	s
γ	23.7 ± 0.2	s
G	16.4 ± 0.7	s
H	14.1 ± 1.1	s
I	12.6 ± 0.6	s
δ	10.6 ± 0.4	s

[1] Calculated from data obtained from 5 independent experiments, in each of which lysates from cells that had been pulse-labelled (15 min), as well as from cells that had been pulse-labelled (15 min) and then chased (105 min), were analyzed. Values are in thousands.

[2] s = stable (no loss of counts during chase period); u = unstable.

when re-initiation can occur and the distribution of ribosomes along the message is uniform. If, as is currently believed, all picornaviral polypeptides are cleaved from a single precursor (Jacobson et al, 1970; Baltimore, 1971; Kiehn and Holland, 1970), the synthesis of radioactive polypeptides will be inhibited in a linear fashion when initiation is blocked. Thus, when the action of pactamycin and the incorporation of radioactive amino acids begins simultaneously, the value of the pactamycin:control ratio (the amount of radioactivity incorporated into a polypeptide during the runoff period relative to the amount of radioactivity incorporated into the same polypeptide under normal steady-state conditions) is proportional to the distance of the gene locus for that polypeptide from the initiation site. The polypeptide synthesized from the 5' end of the message would have the lowest pactamycin:control ratio.

Any delay in the addition of labelled amino acids will decrease the resolution at the 5' end as all pactamycin:control ratios will be very low. If the delay is shorter than the time required to complete synthesis, however, it will increase mapping resolution toward the 5' end.

It should be mentioned that obtaining a linear map by the technique provides further evidence for the existence of a single initiation site in vivo. The presence of a second locus for initiation would result in extensive mapping ambiguity.

Exposure of Mengo virus-infected L. cells to 5×10^{-7} M pactamycin results in immediate inhibition of initiation of protein synthesis. The data presented in Fig. 5 show that the rate of incorporation of radioactive amino acids declines throughout a runoff period of 10 to 12 min

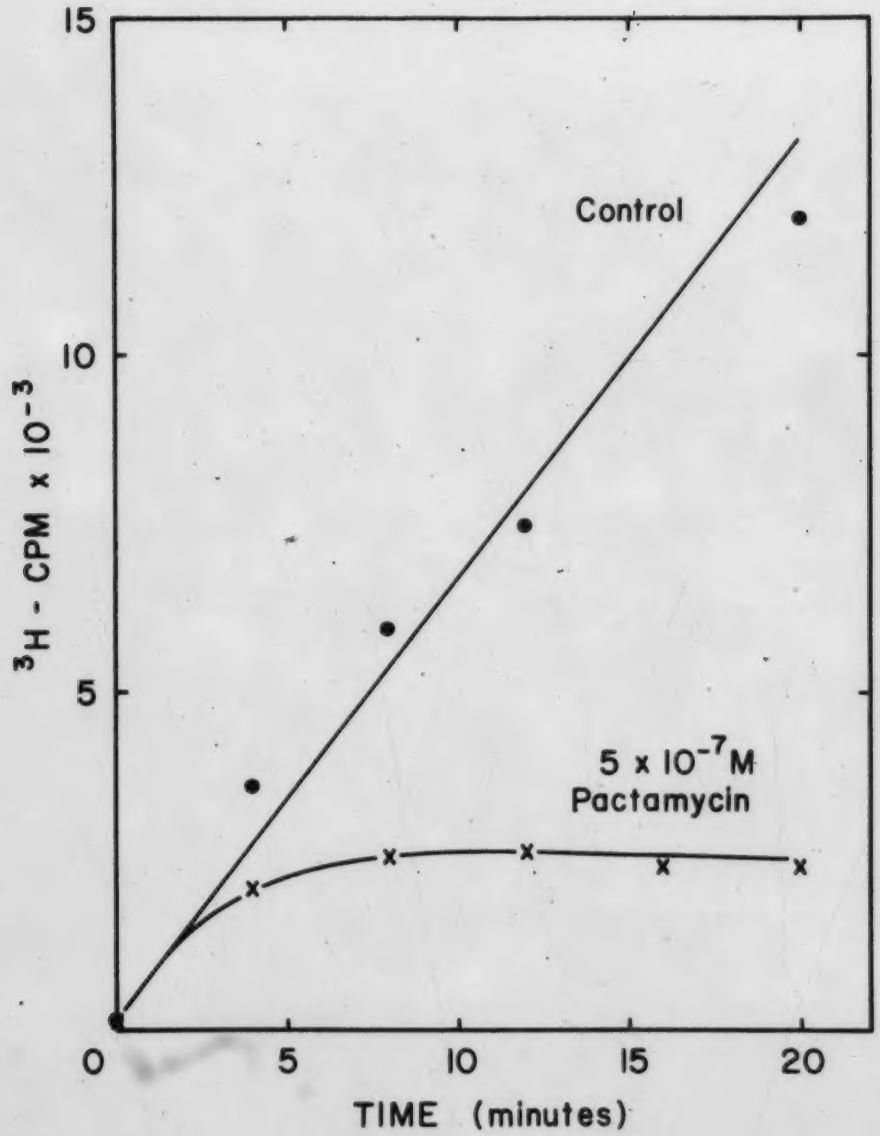


Figure 5. Inhibition of virus-specific protein synthesis by pactamycin. L.cells, grown to confluence on glass cover slips (10.5 x 22mm) were infected with Mengovirus at an estimated moi of 100. At 6 hr p.i. the cells were labelled by the addition of amino acid deficient medium containing 10 μCi/ml ^{3}H-amino acids with or without pactamycin. Duplicate cover slips were removed at the indicated times after addition of label and immersed in chilled 10% TCA for 10 min, then washed sequentially with 5% TCA and 95% ethanol. After air drying the cover slips were immersed in toluene scintillation fluid and the radioactivity in each sample determined in a Beckman Liquid Scintillation Spectrometer.

after administration of the drug, after which time no further synthesis takes place. Therefore, 5×10^{-7} M pactamycin was used to determine the order of synthesis of Mengo specific polypeptides.

The results are summarized in Fig. 6, which shows the apparent gene order of the primary products (upper section) and of the stable products (lower section). The amount of radioactivity in each viral polypeptide in extracts of pactamycin-treated cells was expressed as a fraction of the total radioactivity recovered from all viral polypeptides, and divided by the corresponding figure obtained from SDS-polyacrylamide gel analysis of control (absence of pactamycin) extracts to give the values shown in the figure. The data are strikingly similar to those reported previously by Butterworth and Rueckert (1972a) for EMC virus-specific proteins, the only difference being the inversion of the positions of polypeptides H and I.

The data obtained from the pactamycin studies, considered together with the virtual identity - with respect to both molecular weights and kinetic behavior - of the viral polypeptides of the EMC and Mengo virus systems, suggest strongly that the stable Mengo-specific polypeptides are produced by a pattern of post-translational cleavages very similar to that proposed by Butterworth and Rueckert (1972a) for EMC virus proteins. The scheme consistent with the data presented here is shown in Fig. 7.

To summarize, there are three large primary polypeptides (designated A, F, and C) as well as two smaller, stable, non-capsid polypeptides (G and H) resulting from cleavages of the presumed nascent "polyprotein". Product C, corresponding to the 3' region of the viral RNA, undergoes further proteolytic cleavages to produce the stable,

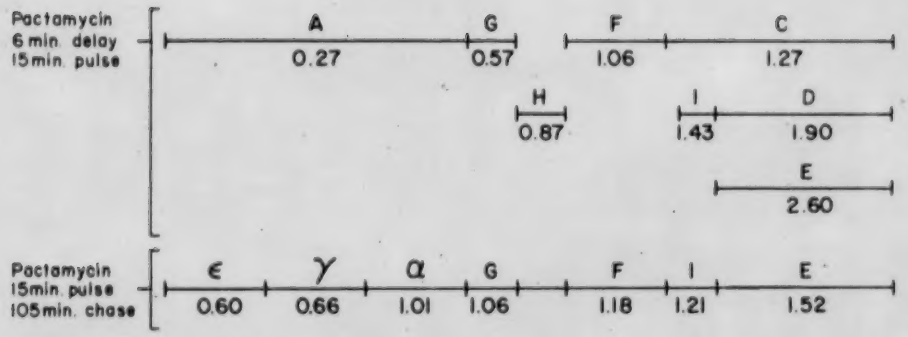


Figure 6. Gene sequence of Mengo virus polypeptides as determined by the pactamycin mapping technique. The numerical values shown in the figure are pactamycin:control ratios, calculated as outlined in the text. Pactamycin was used at a concentration of 5×10^{-7} M.

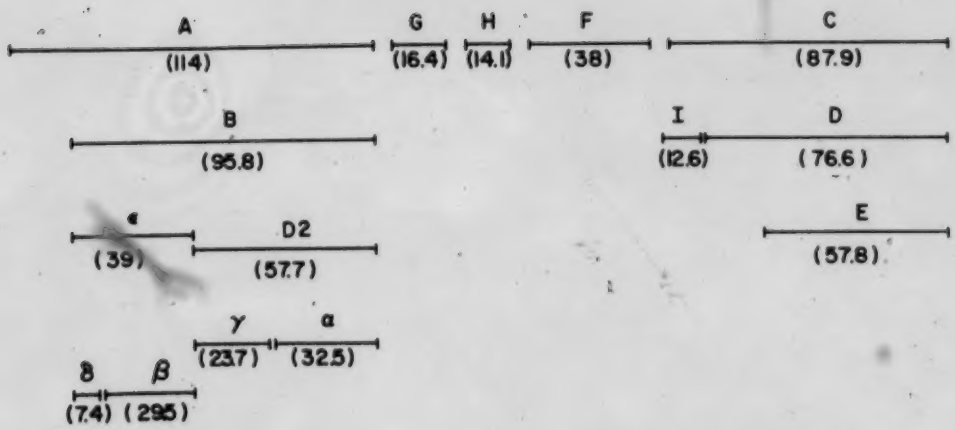


Figure 7. Proposed scheme for the cleavage of Mengo virus-specific polypeptides. The numbers in brackets refer to the molecular weight, in thousands, of each polypeptide.

non-capsid polypeptides E (MW = 57,300) and I (MW = 12,600). Product A, which corresponds to the 5' region of the viral RNA, is the precursor of the capsid polypeptides.

Molar ratios of viral polypeptides

If, as is currently believed, the entire picornavirus genome is translated into a single "polyprotein" (Jacobson et al, 1970; Baltimore, 1971; Kiehn and Holland, 1970) which is cleaved to form the readily detectable primary proteins (i.e. proteins A, F, C), these proteins should be present in infected cells in equimolar amounts, as should the stable end-products derived from them by further proteolytic cleavages. Although, on the basis of published data, this seems clearly to be true of EMC virus-specific polypeptides (Butterworth et al, 1971; Butterworth, 1973), it does not appear to be the case with Mengo virus. In Table 3 are summarized the results of 8 separate experiments in which the amount of each virus-specific polypeptide was quantitated, both in samples prepared from cells that had been pulse-labelled for 15 min and then harvested immediately, and from cells that had been pulse-labelled for 15 min and then incubated in "cold" medium for an additional 105 min. In both cases, the data indicate that, during the pulse-labelling period (6:00 to 6:15 hr post-infection), approximately twice as much capsid as non-capsid protein is synthesized.

The number of cpm in each polypeptide was measured, and expressed as a percent of the total cpm present in all virus-specific polypeptides. These values were then divided by the molecular weights of the polypeptides to give molar ratios, which were normalized relative to the value for polypeptide F, the largest stable product of the primary

TABLE 3

Relative Molar Ratios of Mengo Virus-Specific Polypeptides

Polypeptide	Molecular weight x 10^{-3}	Fraction of total viral cpm[1]		Molar ratio	
		after pulse	after pulse-chase	after pulse	after pulse-chase
A	114.0	0.219(0.039)	-	0.97	-
B	95.8	0.090(0.014)	-	0.48	-
C	87.9	0.066(0.010)	-	0.38	-
D	76.6	0.118(0.011)	-	0.78	-
E	57.3	0.099(0.008)	0.060(0.012)	0.88	0.86
ε	40.0	0.068(0.015)	0.120(0.023)	0.86	0.92
F	38.0	0.075(0.015)	0.124(0.007)	1.00	1.00
α	32.5	0.081(0.016)	0.179(0.014)	1.26	1.69
β[2]	20.5	-	0.112(0.023)	-	1.16
γ	23.7	0.059(0.011)	0.157(0.019)	1.26	2.03
G	16.4	0.068(0.022)	0.097(0.006)	2.10	1.81
H	14.1	0.030(0.007)	0.024(0.003)	1.08	0.51
I	12.6	0.030(0.007)	0.040(0.013)	1.21	0.97
δ[2]	10.6	-	0.027(0.006)	-	0.78
A(total)[3]				2.58	1.93
F				1.00	1.00
C(total)[4]				2.04	0.86

[1]Calculated from data obtained from 8 experiments. Standard deviations are shown in parentheses.

[2]Polypeptides β and δ are not present in lysates prepared immediately following a 15 min pulse.

[3]The total amount of protein A synthesized was calculated from A(total)= $A+B+(\frac{\varepsilon+\alpha+\gamma}{3})$ after a 15 min pulse, and from A(total) = $\frac{(\varepsilon+\beta)+\alpha+\gamma}{3}$ after a pulse followed by a chase.

[4]The total amount of protein C synthesized was calculated from C(total)= C+D+E after a 15 min pulse. After a pulse followed by a chase, the molar ratio of C was considered to be equal to the molar ratio of E.

cleavage. The total amounts of proteins A and C synthesized during a 15 min pulse were calculated - as indicated in the footnote to Table 3 - by summing the amounts of these proteins and the amounts of their cleavage products. In the case of analyses of lysates of cells that had been incubated for 105 min in the absence of labelled amino acids after a 15 min pulse, the amounts of A and C synthesized were calculated from the amounts of the stable end-products derived from them.

As may be seen from the data in Table 3, the molar ratio of protein A:protein F after a 15 min pulse was found to be about 2.5, and to be approximately 2 in lysates of cells after a 15 min pulse and a 105 min chase. With respect to the molar ratio of protein C:protein F, the value based on analyses of lysates made immediately after a 15 min pulse is subject to considerable uncertainty by virtue of the co-migration of polypeptides E and D2 (the immediate precursor of structural polypeptides α and γ).

The presence of D2 in the E peak has been verified by immune precipitation of infected cell lysates with γ-globulins from antiserum against virus capsid polypeptides. Electropherograms of the supernatant and the immune precipitate obtained in one such experiment are shown in Fig. 8, from which it may be seen that immune precipitation removed a significant proportion of the radioactivity from peak E (as well as from other peaks corresponding to capsid polypeptides and their precursors) without reducing the amount of radioactivity in peaks corresponding to other non-structural polypeptides. However, attempts to obtain a <u>precise</u> estimate of the relative amounts of polypeptides D2 and E present in this peak by this method were unsuccessful. Values obtained from one experiment to another were variable, due either to

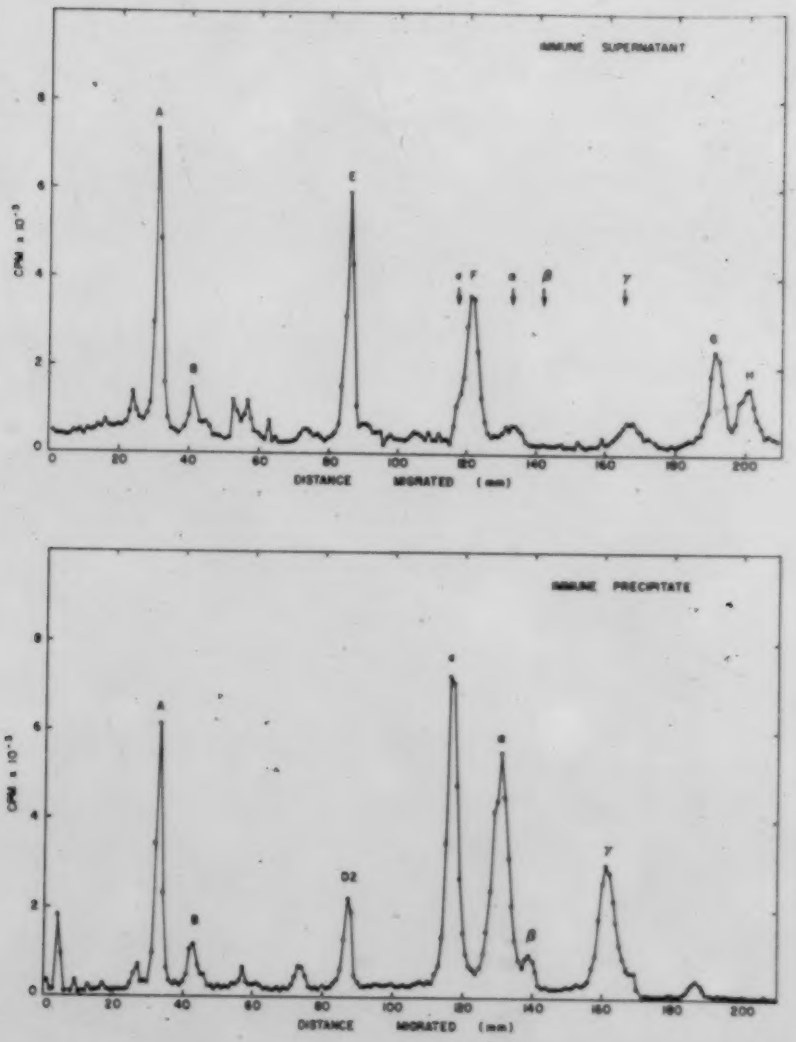


Figure 8. Immune precipitation of Mengo viral capsid proteins from extracts of infected cells. Analysis on SDS-7.5% polyacrylamide gels of the immune precipitate and of the remaining supernatant obtained after incubation of a lysate of Mengo virus-infected L.cells with antiserum to intact virus. Upper panel: supernatant; lower panel: immune precipitate.

some real variability in the relative amounts of these two polypeptides present in different lysates or to variability in the efficiency of precipitation of D2 from one lysate to another (the precipitation of the capsid polypeptides - but not of capsid precursors - by immune γ-globulin was close to quantitative). This being the case, no correction factor was applied to calculations of the molar ratio of protein C: protein F in lysates made immediately after a pulse, and the value of 2.04 cited in Table 3 is too high. However, the value of the ratio C:F obtained from analyses of stable polypeptides is reliable since no D2 remains in the E peak following a chase period (data not shown). Based on such data, the value of this ratio is close to unity.

Polypeptide G is the only non-capsid protein whose molar amount, relative to F, approximates that of the capsid proteins in both situations. H, the other small non-capsid polypeptide produced by primary cleavage of the polyprotein, is found initially in molar quantities essentially equal to F, but appears to be subsequently degraded.

While the high molar ratio of G to F could be due to co-migration of another polypeptide with G, it is unlikely that the elevated level of capsid relative to non-capsid polypeptides can be explained on similar grounds since all major capsid proteins are found in amounts nearly double that of polypeptide F.

These results suggest that the synthesis of Mengo virus-specific polypeptides may be subject to some kind of control late in the replicative cycle of the virus. These considerations prompted an investigation of the synthesis of virus-specific polypeptides throughout the replicative cycle of Mengo virus.

Synthesis of viral-polypeptides during the replicative cycle of Mengo virus

Infected L. cells were pulse-labelled for 15 min at hourly intervals from 3 to 6 hr p.i., and then lysed either immediately or after a chase period of 105 min. Lysates were analyzed for virus-specific polypeptides by electrophoresis in SDS-7.5% polyacrylamide gels as described earlier. Representative electropherograms are presented in Fig. 9.

Electrophoretic profiles obtained with lysates prepared 3 hr p.i. were found to be essentially the same as those obtained with lysates of uninfected cells, and are not shown here. Between 3 and 4 hr p.i. however, there is a fairly abrupt shut-off of cellular protein synthesis, so that by 4 hr p.i. the virus-specific polypeptides may be identified unambiguously. Thereafter, the background of cellular protein synthesis decreases progressively and the synthesis of virus-specific polypeptides predominates.

The relative molar quantities of the various polypeptides were calculated as described previously.

The essential findings arising from this study are summarized in Table 4. Shown here are estimates of the amounts of capsid protein, A(total), of non-capsid protein, C(total), synthesized at various times p.i. and expressed relative to the amount of polypeptide F. The value of the ratio A(total):F was found to increase progressively from close to unity at 4 hr p.i. to 2 or more late in the infectious cycle. The same trend was observed in the case of the ratio G:F. It should be recalled that the values of the ratio C(total):F calculated from electropherograms of lysates made immediately after a 15 min pulse are

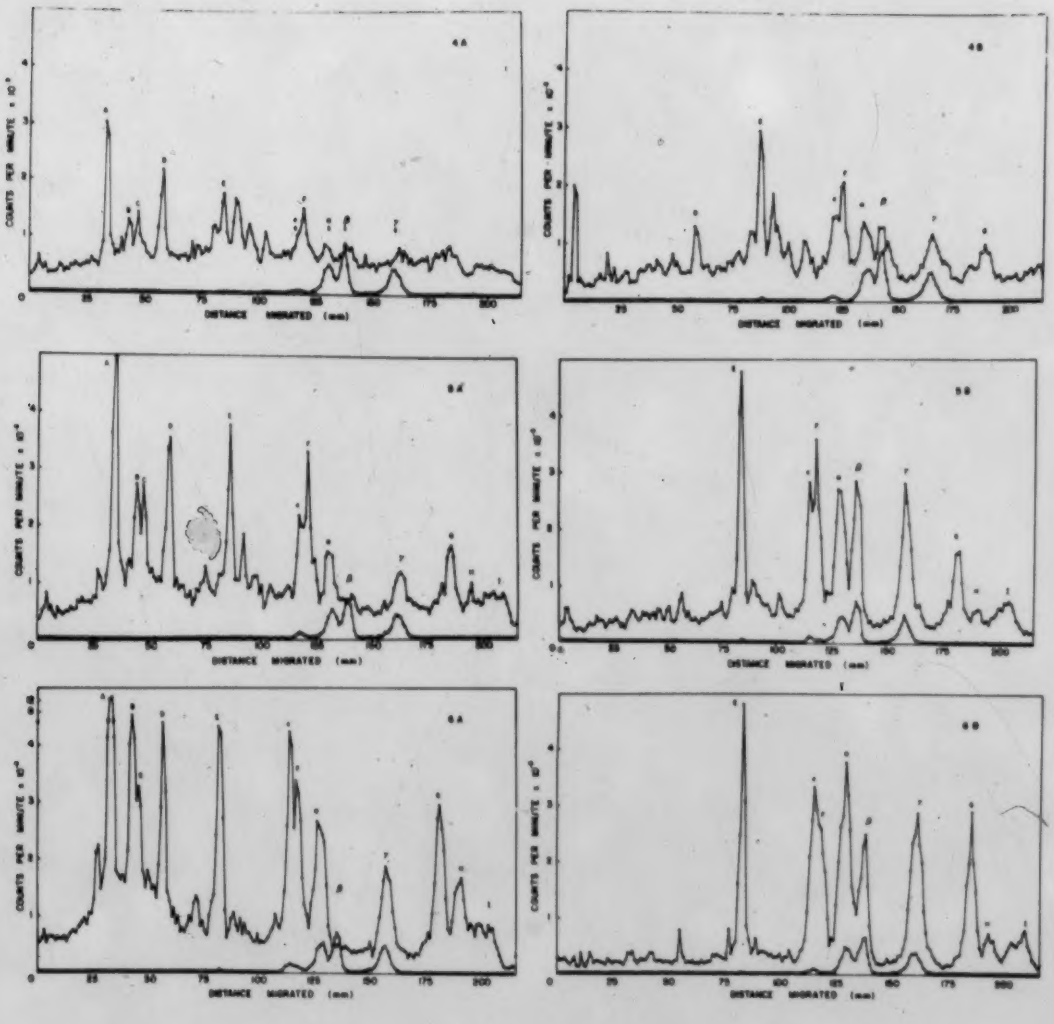


Figure 9. Electrophoresis in SDS-7.5% polyacrylamide gels of lysates of Mengo virus-infected L.cells, pulse labelled for 15 min with ^{3}H-amino acids at 4, 5 and 6 hr post-infection. Series A: cells were lysed immediately after the labelling period. Series B: cells were lysed after a chase period of 105 min. The lower profile in each panel is given by the polypeptides of purified, ^{14}C-amino acid labelled Mengo virions added to each sample prior to solubilization.

TABLE 4

Relative Molar Ratios of Mengo Virus-Specific Polypeptides

Viral polypeptide(s)	Molar ratio[3] after pulse given at					
	4 hr p.i.		5 hr p.i.		6 hr p.i.	
	After pulse	After pulse-chase	After pulse	After pulse-chase	After pulse	After pulse-chase
EXP.1						
A(total)[1]	1.20	1.28	1.43	1.71	2.95	2.50
F	1.00	1.00	1.00	1.00	1.00	1.00
C(total)[2]	1.07	1.08	1.26	0.89	1.62	0.89
EXP.2						
A(total)	0.63	1.08	1.15	1.25	2.10	1.86
F	1.00	1.00	1.00	1.00	1.00	1.00
C(total)	1.10	0.85	1.29	0.90	1.76	1.00

1. The total amount of protein A synthesized was calculated from $A(total) = A + B + [(\varepsilon + \beta + \alpha + \gamma)/3]$.

2. The total amount of protein C synthesized was calculated from $C(total) = C + D + E$.

3. Values for A(total) and C(total) are normalized with respect to F.

distorted due to the presence of significant amounts of polypeptide D2 (the immediate precursor of the capsid polypeptides α and γ) in peak E. The values for this ratio obtained from analyses of lysates made after a pulse followed by a chase were found to remain close to unity at all times examined.

The progressive increase in the ratio of capsid to non-capsid protein synthesized as infection proceeds is compatible with the proposal that the translation of the Mengo virus genome may be subject to some kind of control.

The simplest and most obvious explanation of the phenomenon described here is that there is a rapid and selective degradation of non-capsid polypeptides late in the infectious cycle. This possible explanation appears to be ruled out by the observation that (i) the ratio of capsid polypeptides:polypeptide F (the reference non-capsid polypeptide) after a 15 min pulse is essentially the same as after a 15 min pulse followed by a 105 min chase, and (ii) the non-capsid polypeptides E and F, synthesized during a 15 min pulse period, persist in approximately equimolar and essentially undiminished amounts during relatively long periods of time subsequent to the pulse. Lucas-Lenard (1974) has also presented kinetic evidence indicating that polypeptide F is stable. It seems more likely from these observations that more capsid than non-capsid protein is synthesized.

In view of the strong evidence that picornaviral genomes contain a single site for the initiation of translation, the most likely model to explain the asymmetric translation of the viral RNA is one in which some viral specified factor acts as a specific termination factor capable of causing premature termination of translation at a site

located near the midpoint of the viral RNA.

Effect of amino acid analogues on synthesis of viral proteins

Jacobson et al (1970) have shown that the presence of the amino acid analogues p-fluorophenylalanine, ethionine, canavanine (an arginine analogue) and azetidine-2-carboxylic acid (a proline analogue) blocks the cleavage of high molecular weight polypeptides synthesized during the replication of poliovirus in HeLa cells, and thus permits the detection of the putative translation product of the entire viral genome. Comparable experiments, using these same analogues, were carried out with the Mengo virus-L. cell system in an effort to detect precursors of virus-specific polypeptides larger than those identified previously (see Fig. 3).

Cells were pulse-labelled for 30 min (from 6:00 to 6:30 hr post-infection) and lysates, made immediately thereafter, were analyzed by electrophoresis in SDS-5% polyacrylamide gels. The results are illustrated in Fig. 10 (panel B), from which it may be seen that, in addition to protein A, three proteins of molecular weights greater than that of A and here designated as X, Y and Z, were found. The molecular weights of these proteins, were determined from their rates of migration in SDS-5% polyacrylamide gels relative to those of high molecular weight marker proteins as shown in Fig. 11. These molecular weights are presented in Table 5.

Protein Z, with a MW of about 200,000, is analogous to the "polyprotein", NCVP·00, found by Jacobson et al (1970) and by us (see Fig. 17, Chapter IV) in HeLa cells infected with poliovirus under these conditions, and may represent the product of translation of the

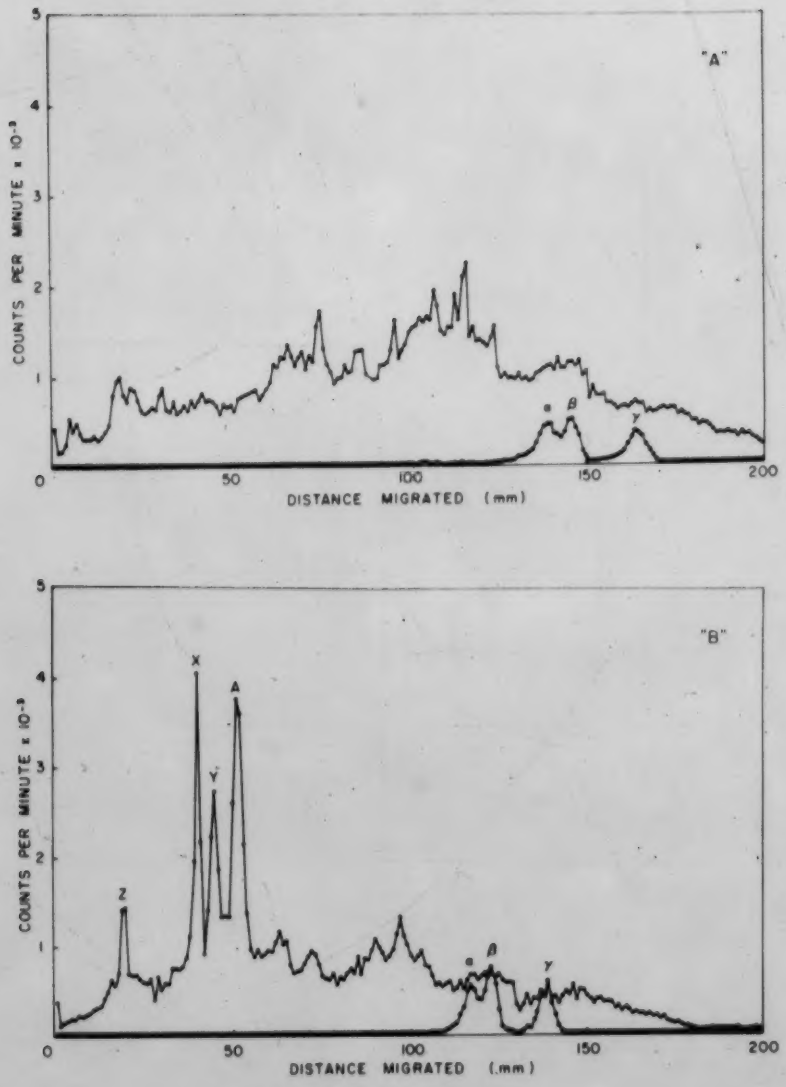


Figure 10. Profiles obtained from electrophoresis in SDS-5% polyacrylamide gels of lysates of L.cells, pulse-labelled with ^{3}H-amino acids in the presence of the amino acid analogues p-fluorophenylalanine, ethionine, canavanine and azetidine-2-carboxylic acid. The positions of the polypeptides derived from purified, ^{14}C-amino acid labelled Mengo virions are shown by the closed circles. Panel A: Mock-infected L.cells, pulse-labelled for 30 min at 6 hr post-infection. Panel B: Mengo virus (M-variant)-infected L.cells, pulse-labelled for 30 min at 6 hr post-infection.

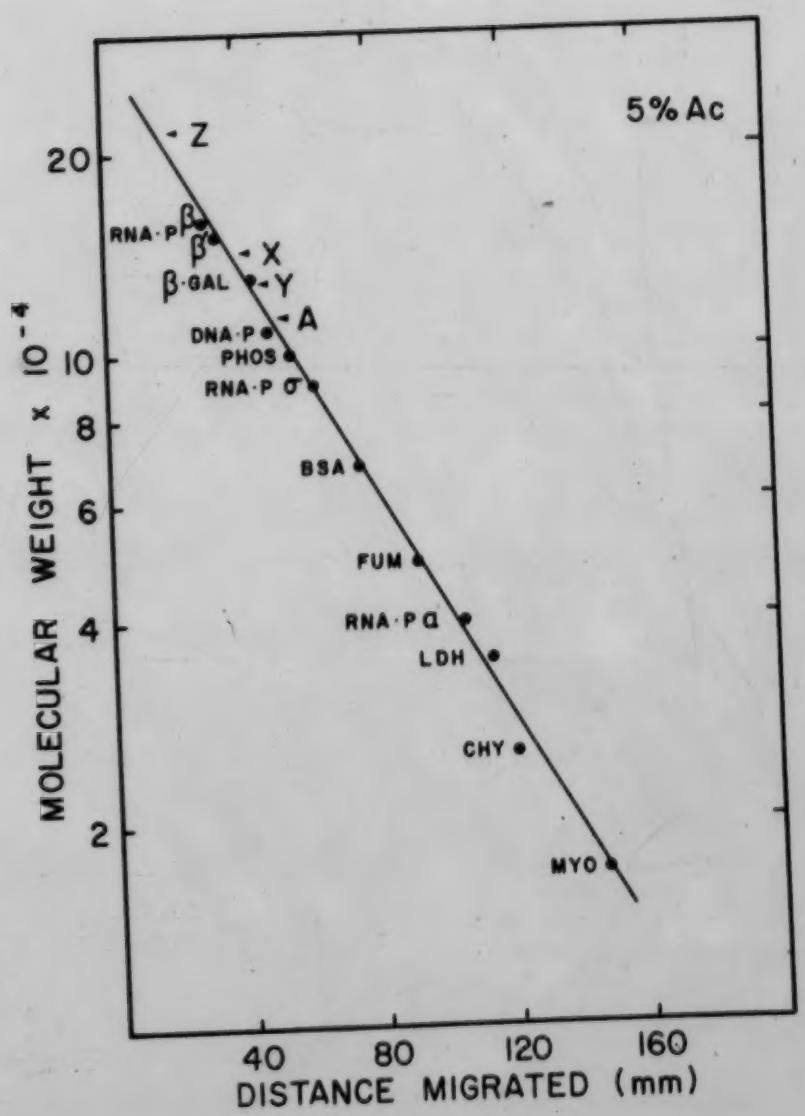


Figure 11. Molecular weights of Mengo virus-specific polypeptides synthesized in the presence of amino acid analogues as determined from migration in SDS-5% polyacrylamide gels. The molecular weights of the marker polypeptides are listed on p38 . ● = position of marker polypeptide, ◄ = position of viral polypeptide.

TABLE 5

Molecular Weights and Molar Ratios of Mengo Virus-Specific Polypeptides Synthesized in the Presence of Amino Acid Analogues

Polypeptide	Molecular weight[1]	Fraction of total viral cpm	Molar ratio[2]
Z	216.0 ± 7.8	0.112	0.15
X	142.5 ± 6.5	0.256	0.52
Y	126.8 ± 7.6	0.238	0.55
A	114.0 ± 4.3	0.394	1.00

[1] $\times 10^{-3}$

[2] Molar ratios of polypeptides X, Y and Z are normalized with respect to A.

entire Mengo virus genome. Although the molecular weight of Z is approximately twice that of protein A, it is unlikely that it is a dimer of A because neither heating the lysate (5 min, 100°) with 6 M urea prior to electrophoresis, nor electrophoresis in the presence of 6 M urea had any effect on the size or shape of the peak. The possibility that Z is a cellular protein, whose synthesis is induced by virus infection, is more difficult to rule out, but on the basis of the previously cited studies of poliovirus replication, and despite the fact that it is present in such small amounts, it seems more likely that it does correspond to the Mengo virus-specific "polyprotein".

It is not possible to draw any conclusions regarding the nature of polypeptides X and Y from these data. The molecular weight of X (142,500 ± 6,500) corresponds, within the limits of experimental error, to the combined molecular weights of either proteins F, C and H (140,000 ± 4,200) or proteins A, G and H (144,500 ± 5,800). By the same token, polypeptide Y (MW = 126,800 ± 7,600) could be considered to be composed of either polypeptides A and G (combined MW = 130,400 ± 4,700) or F and C (combined MW = 125,900 ± 3,100).

The results of immune precipitation studies (data not shown) did not resolve the problem of identification, in that immune γ-globulins against the capsid polypeptides were found to precipitate a fraction of the radioactivity found in both peaks X and Y. The amount that was precipitated varied, in different experiments, from 20% to 50% (which is equivalent to the extent to which protein A, synthesized in either the presence or absence of amino acid analogues, is precipitated by the same immune γ-globulins). Neither of the non-capsid polypeptides

F or C is precipitated to any extent by these γ-globulins. It seems possible that there is some heterogeneity in the protein present in the two peaks, but very probable that both contain a substantial proportion of protein containing capsid polypeptide sequences.

Effect of amino acid analogues on synthesis of viral polypeptides throughout the replicative cycle

The results of studies of the synthesis of viral-specific polypeptides in the absence of amino acid analogues showed that during the replication of Mengo virus the genome is translated asymmetrically, and that the degree of asymmetry increases progressively as a function of time p.i. According to the model proposed to explain these observations, the viral genome would be translated *in toto* early in the infectious cycle. As the cycle progressed, however, premature termination of translation at a site located near the midpoint of the viral RNA would result in a progressive decrease in the synthesis of non-capsid proteins, the information for which is located toward the 3' end of the RNA, and a corresponding increase in the relative amount of capsid proteins which are translated from the 5' end of the genome. When Mengo virus-infected cells were pulse-labelled in the presence of amino acid analogues, the cell lysates were shown to contain protein Z (believed to be the product of translation of the entire viral genome) and protein A (the precursor of all four capsid polypeptides), as well as proteins X and Y of uncertain identity. It was reasoned that if the model is correct, a progressive decrease should be seen in the ratio of 'polyprotein' Z: capsid protein A as a function of time p.i. when cells are pulse-labelled in the presence of amino acid analogues.

This proposition was tested by analyzing lysates of infected cells, pulse-labelled for 30 min in the presence of amino acid analogues at half-hourly intervals from 4 to 6 hr p.i. Illustrative electropherograms are shown in Fig. 12, from which it may be seen that there is a progressive decrease in the amount of protein Z relative to protein A synthesized as a function of hr p.i. The phenomenon may be more clear from the data presented in Table 6, in which the molar ratio of polyprotein Z (as well as those of proteins X and Y) relative to capsid precursor A at various times p.i. are listed. The value of the molar ratio of Z:A was found to decrease progressively from 4 to 6 hr p.i., and observation consistent with the proposed model for control of translation. No consistent changes were observed in the relative molar ratios of polypeptides X and Y, and interpretation of the data regarding these components is difficult since their polypeptide composition has not been determined.

Discussion

The Mengo virus genome, of molecular weight 2.44×10^6 (Ziola and Scraba, 1974) can code, theoretically, for a maximum of about 260,000 daltons of protein, and since the sum of the molecular weights of the primary translation products (identified in this investigation) is very close to this value, it seems reasonable to assume that all virus-specific polypeptides have been identified. The pattern of post-translational cleavages leading to the production of stable viral gene products in the Mengo virus system is very similar to that previously described for EMC virus. Apart from some minor differences in the estimated molecular weights of corresponding polypeptides and in the

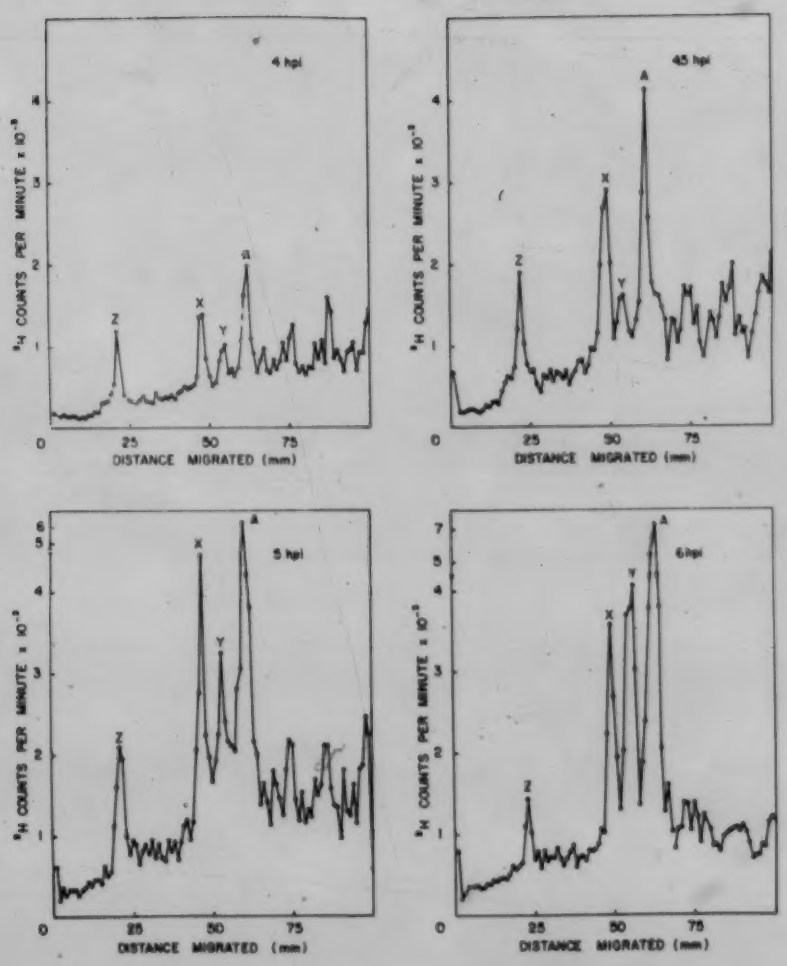


Figure 12. Electrophoresis in SDS-5% polyacrylamide gels of lysates of Mengo virus-infected L.cells, pulse labelled for 30 min in the presence of amino acid analogues, at 4.0, 4.5, 5.0 and 6.0 hr p.i.

TABLE 6

Relative Molar Ratios of Mengovirus-Specific Polypeptides Produced in the Presence of Amino Acid Analogues

Polypeptide	Molecular weight x 10^{-3}	Molar ratio[1.] after pulse given at				
		4hr p.i.	4.5hr p.i.	5hr p.i.	5.5hr p.i.	6hr p.i.
Z	220	0.24	0.23	0.18	0.12	0.09
X	142	0.52	0.74	0.42	0.43	0.37
Y	126	0.56	0.37	0.54	0.54	0.58
A	114	1.00	1.00	1.00	1.00	1.00

1. Values for X, Y and Z are normalized with respect to A.

kinetics of their interconversion, data obtained from studies of the two systems differ significantly in only two respects. First, lysates of Mengo virus-infected cells do not appear to contain a protein analogous to the EMC virus-specific polypeptide D1, believed to be a precursor of polypeptides ε and γ, and thus to be an alternate cleavage product of B at the level of D2 (see Fig. 7). Second, the results of the pactamycin studies suggest that the segment of the viral genome that codes for polypeptide H is closer to the 5' end than that which codes for polypeptide I, and the estimate of the molecular weight of polypeptide H makes it seem unlikely that it is derived, together with D, by cleavage of C. A cleavage scheme compatible with these observations is one in which polypeptide H is formed by primary cleavage of the nascent "polyprotein", and polypeptides I and D are produced by cleavage of C.

The data presented here show that capsid and non-capsid viral polypeptides are not present in equimolar amounts in infected cells late in the replicative cycle, and suggest that their synthesis may be subject to some kind of translational control.

The possibility that the elevated level of capsid polypeptides (relative to non-capsid polypeptides) is due to co-migration of capsid and host cell polypeptides is very unlikely, since all major polypeptides - ε(including β and δ) α and γ - are found in amounts nearly double that of polypeptide F. Such an explanation would require the assumptions that (i) the cell contains polypeptides of molecular weights identical to those of the capsid polypeptides, and (ii) the synthesis of these polypeptides (unlike that of other cell proteins) is not shut off by virus replication. This explanation is also rendered untenable by the observation that the molar ratio of capsid:non-capsid

polypeptides increases throughout infection from unity to two or greater late in the replicative cycle. This progressive increase also speaks strongly in favor of the existence of some specific control process.

Since the stability of polypeptide F argues against rapid and selective degradation of non-capsid polypeptides in this system, it seems likely that more capsid than non-capsid protein is synthesized.

It is possible to imagine two ways in which control of translation might be exerted in this system. First, one could speculate that the Mengo genome has two or more initiation sites for translation, as is the case with some RNA phages (Weissman et al, 1973) and that initiation of translation of that segment of the genome which codes for the capsid polypeptides is more efficient than that of the other segment or segments. This seems unlikely for several reasons. Öberg and Shatkin (1972, 1974) have shown that in vitro Mengovirus RNA possesses only one site for initiation of translation. That this is also true in vivo is suggested by the studies with pactamycin; a reasonable map could not be obtained if translation began at two sites on the genome. Evidence obtained from studies of the poliovirus system indicates that the genome of this virus is translated into a giant polypeptide that serves as a precursor for all the virus-specific polypeptides produced during infection (Roumiantzeff et al, 1971; Korant, 1972; Garfinkle and Tershak, 1971). The studies with amino acid analogues reported by Jacobson et al (1970) and repeated by us (see Fig. 17, Chapter IV) show the accumulation of large amounts of NCVP·00, the candidate for this polyprotein precursor in poliovirus-infected cells. The presence of protein Z in Mengo virus-infected cells, albeit in small quantities, lends credence to the hypothesis that the biosynthesis of Mengo- and poliovirus poly-

peptides proceed by similar, if not identical, mechanisms.

It is difficult to picture a mechanism by which an internal initiation site would be utilized under some conditions, but read through under others. On the other hand, control of translation by specific termination is not only easier to envision but easier to reconcile with the data presented here, and could be accomplished if some viral protein (or proteins) functioned as a termination factor (or factors). According to this model, the viral genome would be translated _in toto_ early in the infectious cycle. As the cycle progressed, however, premature termination of translation at a site located near the midpoint of the viral RNA would result in a progressive decrease in the synthesis of non-capsid proteins, the information for which is located toward the 3' end of the RNA, and a corresponding increase in the relative amount of capsid proteins which are translated from the 5' end of the genome.

While premature termination at discrete sites near the middle of the genome is a well documented feature of studies of translation of cardioviral RNAs _in vitro_ (Kerr _et al_, 1972; Boime and Leder, 1972; Öberg and Shatkin, 1972; Eggen and Shatkin, 1972; Smith, 1973), it is difficult to demonstrate _in vivo_. The model predicts that as termination becomes more frequent (late in infection) the amount of polyprotein synthesized during a discrete time interval should decrease relative to the amount of capsid precursor A made at the same time. The data arising from an examination of the viral-specific polypeptides synthesized in the presence of amino acid analogues, and presented in Fig. 12 and Table 6, are compatible with the model without providing conclusive evidence for it. The value of the molar ratio of Z:A does decrease progressively throughout the infectious cycle as predicted.

The synthesis of polypeptides X and Y, neither of which has been identified unambiguously, makes interpretation of the data difficult. However, the finding that both are precipitated, at least in part, from lysates by antibodies against the virus capsid polypeptides suggests that polypeptides X and Y may consist of polypeptides A + G and A + G + H, respectively, and that would support the proposed model.

An obvious, but not the only, candidate for the polypeptide exercising the hypothetical control function is G, which is synthesized in amounts equimolar to those of the capsid polypeptides, but is not incorporated into Mengo virions. This suggestion is given some credibility by the reports (Matthews et al, 1973; Medvedkina et al, 1974) that significant amounts of polypeptide G are associated with ribosomes in EMC virus-infected cells. However, convincing evidence will probably come only from an examination of the effects of individual, isolated non-capsid proteins on the pattern of synthesis of viral-specific polypeptides in a cell-free system.

While no mechanism of translational control in picornavirus systems has been suggested previously, it is intellectually satisfying, in that it would impose a degree of energetic economy late in the infectious cycle when capsid proteins are required in large amounts for the assembly of progeny virions, while non-capsid polypeptides, which probably have catalytic functions, are less in demand.

IV: COMPARATIVE STUDIES WITH POLIOVIRUS

Introduction

Poliovirus is generally regarded as the prototype of the picornaviruses, since it was employed in most of the early studies of replication of this group of viruses. In 1967, Summers et al reported that although both the size and activity of poliovirus polysomes appear to decrease late in infection, essentially the same spectrum of viral proteins is synthesized throughout the replicative cycle. At the time this study was carried out, it was not possible to quantitate the relative amounts of viral polypeptides synthesized. More recently, Butterworth (1973) described the results of studies of the synthesis of poliovirus (type 2) polypeptides in HeLa cells. He presented a cleavage map for the viral specific polypeptides (based on data obtained by means of the pactamycin mapping technique) and concluded that, during the mid log phase of virus production, capsid and non-capsid proteins are synthesized in 'roughly equimolar amounts'. However, the validity of this conclusion is subject to some uncertainty, since in this system the polypeptides VP·1 (capsid) and NCVP·X (non-capsid) are not resolved by SDS-polyacrylamide gel electrophoresis, the analytical method employed.

In view of the data suggesting that the synthesis of Mengo virus polypeptides is subject to some kind of translational control, and considering the rather equivocal nature of the published data on the poliovirus system, it was considered worthwhile to re-examine the latter to see if some evidence for control could be obtained.

Materials and Methods

Cells and virus

HeLa cells (obtained from the American Type Culture Collection) were grown in suspension culture in calcium-free Eagle's minimum essential medium, and as monolayers in Eagle's basal minimum essential medium, both media supplemented with 5% calf serum.

Poliovirus (W. Chat, Type 1) was obtained originally from Dr. Hilary Koprowski of the Wistar Institute, Philadelphia, Pa., and was plaque-purified before use. It was propagated in HeLa cells and assayed for infectious virus by standard plaque assay HeLa cell monolayers. The inoculum used throughout the studies described here was a relatively crude preparation obtained by sedimenting the virus from clarified lysates of infected HeLa cells by centrifugation for 1 hr at 100,000 xg and then resuspending the pellet in 0.1M sodium phosphate, pH 7.4. ^{14}C-amino acid-labeled poliovirus, used as the source of marker polypeptides in the gel electrophoresis studies, was purified by the method described earlier for Mengo virus except that the initial concentration of virus from clarified lysates was effected by centrifugation (1 hr, 100,000 xg) rather than by methanol precipitation.

Experimental procedures

The methods used for the infection of monolayers, for the labelling of viral polypeptides (in the presence and absence of amino acid analogues), for the preparation of cell lysates, and for SDS-polyacrylamide gel electrophoretic analyses were identical to those described in detail in Chapter III.

Results

The replication of poliovirus proceeds more rapidly than that of Mengovirus. From the single cycle growth curve of poliovirus in HeLa cells shown in Fig. 13, it may be seen that the logarithmic phase of viral production occurs between 3 and 4.5 hr p.i., nearly two hours earlier than in the Mengo system.

Synthesis of poliovirus-specific polypeptides

In order to identify the polypeptides produced during the replication of poliovirus, HeLa cells were pulse-labelled for 15 min with ^{3}H-amino acids at 3.5 hr after infection and lysed either immediately or after a chase period of 105 min. The lysates were analyzed on 7.5% polyacrylamide gel in the presence of SDS as described in Chapter III. Representative electropherograms are shown in Fig. 14. More than 70% of the radioactivity present in samples prepared after a 15 min pulse is found in three large precursor polypeptides designated NCVP·1, NCVP·1 1/2 and NCVP·2 (according to the nomenclature of Baltimore and co-workers; NCVP = non-capsid viral protein) whose molecular weights are 110,000, 100,000 and 80,000 respectively. These correspond, on the basis of molecular weights, to Mengo polypeptides A, B and D respectively. Very little of the non-capsid NCVP·X (which corresponds to F in the Mengo system) and of the capsid polypeptides VP·0, 1 and 3 can be detected in lysates prepared immediately after a 15 min pulse. In lysates prepared after a 105 min chase on the other hand, only small amounts of the precursor proteins NCVP·1 and 1 1/2 are present, the radioactivity contained therein having been chased into the now clearly visible capsid polypeptides. However, unlike the situation with the

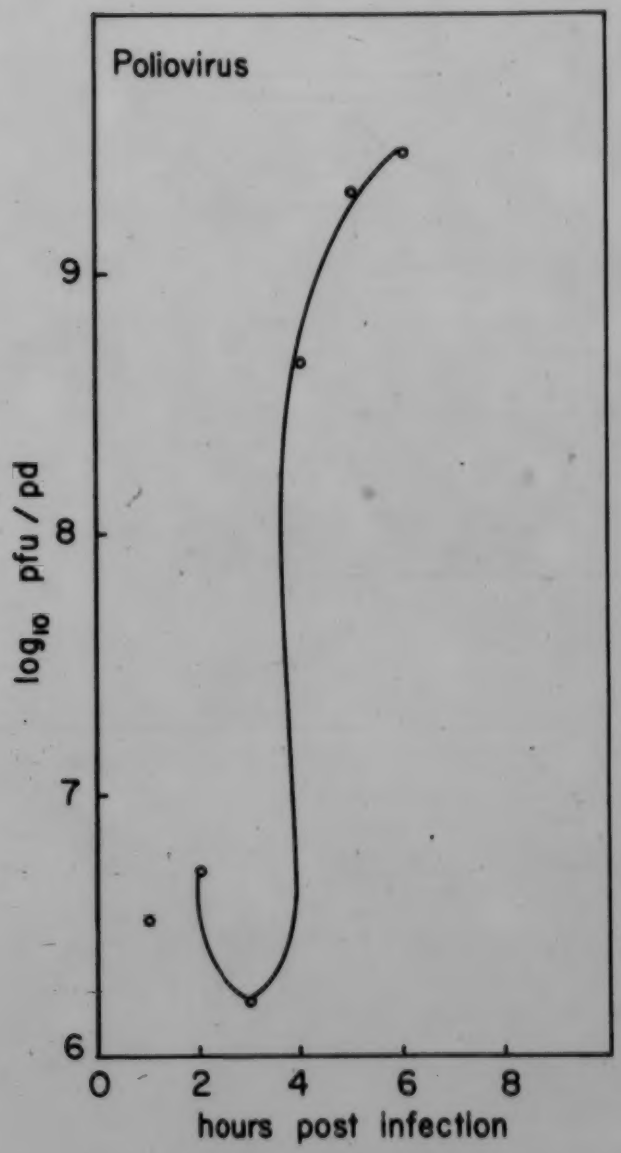


Figure 13. Single cycle growth curve of poliovirus in HeLa cell monolayers. Output is expressed as pfu/60 x 15mm petri dish (∿ 5 x 10^6 cells).

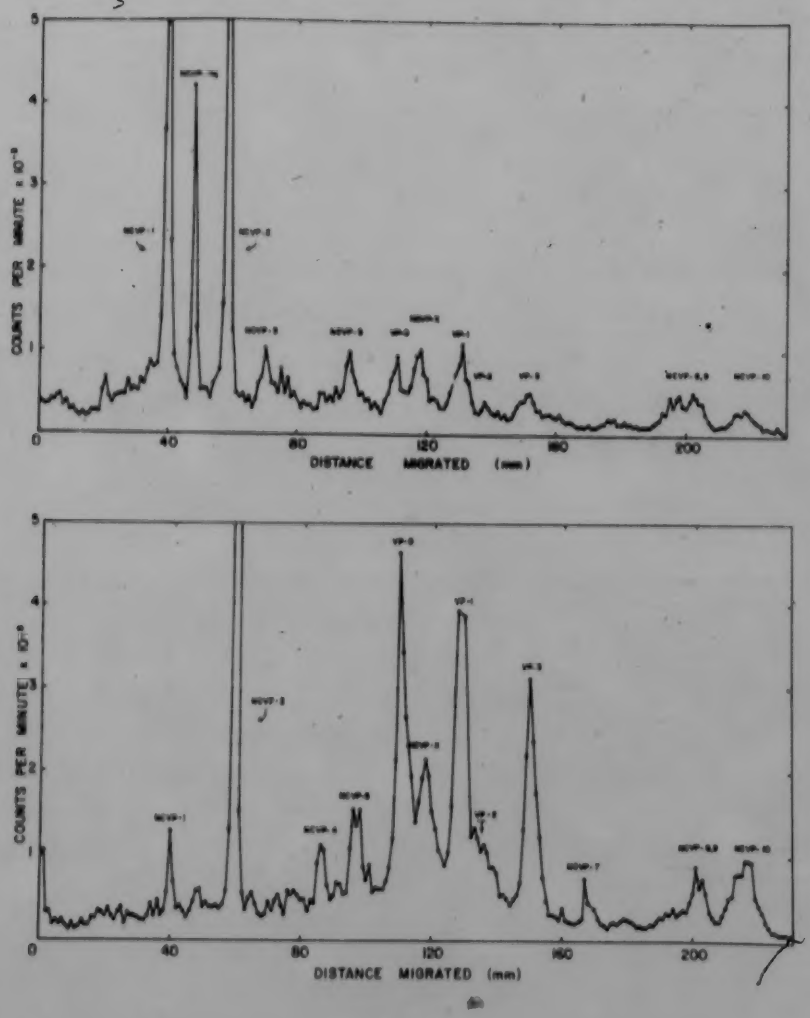


Figure 14. Profiles obtained from electrophoresis in SDS-7.5% polyacrylamide gels of lysates of poliovirus-infected HeLa cells, pulse labelled with ^{3}H-amino acids for 15 min at 3.5 hrp.i. Upper panel: lysate prepared immediately after pulse; lower panel: lysate prepared after a chase period of 105 min.

corresponding Mengo polypeptide D, relatively large quantities of NCVP·2 remain even after a chase. Small peaks of other polypeptides, identified in Fig. 14 as NCVP·3-10, are also found in these gels (there is no NCVP·6 peak, since the polypeptide that was assigned that number in earlier studies was found subsequently to be a capsid precursor and was 'renamed' VP·0).

Cleavage scheme of poliovirus polypeptides

Early work using the antibiotic pactamycin established the gene order of the primary polypeptides of poliovirus to be (5' → 3') NCVP·1 - NCVP·X - NCVP·2 (Taber et al, 1971; Summers and Maizel, 1971). That NCVP·1 is the precursor of the capsid polypeptides had been determined earlier by tryptic fingerprinting of isolated protein (Jacobson et al, 1970). Using the pactamycin technique, Butterworth (1973) constructed a more complete cleavage map of the polypeptides of Type 2 poliovirus. This has been modified to take into account the molecular weights of the Type 1 poliovirus polypeptides determined from the present study, and is presented in Fig. 15. Polypeptide NCVP·1 is the precursor of the capsid polypeptides VP·1 to 4. NCVP·1 1/2 is generally considered to be the largest precursor of the non-structural viral polypeptides. However, the molecular weight of this polypeptide in our system differs substantially from that published by Butterworth (1973), which makes its position in the overall cleavage scheme uncertain. It is quite possible, for example, that NCVP·1 1/2 actually corresponds to the Mengoviral polypeptide B, in its position in the cleavage scheme as well as in molecular weight, and that it is derived from NCVP·1, the capsid precursor. Because of the uncertainty regarding at least some of the

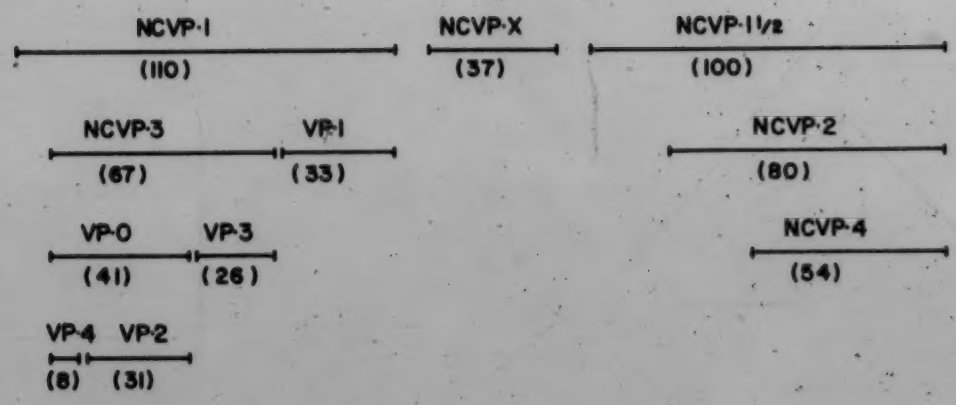


Figure 15. Proposed scheme for cleavage of poliovirus polypeptides (adapted from Butterworth, 1973). The numbers in brackets refer to the molecular weights of the polypeptides (in thousands) determined from the present study.

precursor-product relationships, all subsequent quantitative studies were conducted on lysates prepared after a chase period, in which only the stable viral polypeptides were radiolabeled.

The positions of the minor polypeptides NCVP·5 to 10 in this cleavage scheme are unclear. Data obtained from studies with pactamycin (Butterworth, 1973) indicate that they are translated from the central portion of the genome, that is, from that segment between those coding for VP·3 and NCVP·4 (see Fig. 15). The smaller fragments may be the products of normal cleavage. However, poliovirus precursor proteins appear to possess multiple cleavage sites, which may give rise to ambiguous cleavages. For example, on high resolution gels, the peaks corresponding to the capsid polypeptides VP·2 and VP·3 can be shown to contain two or three separable species (Cooper et al, 1970; van den Berghe and Boeyé, 1972; Phillips and Fennel, 1973). Thus NCVP·5 to 10 may be either products of ambiguous cleavages of non-structural polypeptides or degradation products of such incorrectly cleaved polypeptides. The molecular weight differences among analogous polypeptides in various strains of poliovirus may be explained by differences in susceptibility to cleavage at such ambiguous sites.

Molar ratios of the viral polypeptides

In order to quantitate the amounts of the major polypeptides produced by poliovirus, relative molar ratios were calculated as described for Mengo virus. The molar ratios were normalized with respect to NCVP·X (MW = 37,000; the poliovirus counterpart of Mengo polypeptide F) which in our system is clearly resolved from VP·1 (MW = 33,000). The results are summarized in Table 7. Also listed is

TABLE 7

Relative Molar Ratios and Molecular Weights of the Major Poliovirus Polypeptides[1.]

Poliovirus polypeptide	Corresponding Mengovirus polypeptide	Molecular weight x 10^{-3}	Molar ratio[2.]
NCVP·1	A.	110	0.11
NCVP·1 1/2	Bor C.	100	--[3.]
NCVP·2	D.	80	0.53
NCVP·4	E.	54	0.18
VP·0	ε.	40	1.90
NCVP·X	F.	37	1.00
VP·1	α.	33	2.19
VP·3	γ.	26	1.84

1. From analysis of a lysate of HeLa cells, pulse labeled for 15 min at 3.5 hr p.i. (mid-logarithmic phase of virus production) and chased for an additional 105 min.

2. Normalized with respect to NCVP·X. Each value is the average from three independent experiments that gave very similar results. Average deviation from these mean values was 0.15 with a maximum (VP·1) of 0.22.

3. Not calculated since the NCVP·1 1/2 peak is ill-defined.

the Mengo polypeptide which corresponds to each of the polio polypeptides, together with the molecular weight of the latter. The data show that the molar ratio of each of the capsid polypeptides (VP·0, VP·1, VP·3):NCVP·X is equal to approximately 2 at this time (3.5 hr p.i.) in the replicative cycle. The cleavage of NCVP·2 is obviously much slower than is the corresponding conversion of D → E in the Mengo system since much of the precursor is still present after the chase period. The sum of the molar ratios of NCVP·2 and NCVP·4, which by analogy with the Mengo system might be expected to equal that of NCVP·X, is only 0.71. It seems likely that this low value reflects the instability of NCVP·4. The molar ratios of the minor polypeptides which were found to be variable and to have values less than 0.2, are not included in the table.

These data show that in this system, as in the Mengo system, nearly twice as much capsid as non-capsid protein is present late in infection, and prompted an examination of the synthesis of poliovirus polypeptides throughout the replicative cycle of poliovirus.

Synthesis of polypeptides throughout the replicative cycle of poliovirus

The total amount of capsid protein (Ca) synthesized at various times throughout the replicative cycle of poliovirus was estimated from electropherograms, such as those illustrated in Fig. 16, obtained from lysates of infected cells pulse labelled for 15 min at 2.5, 3.0, 3.5 and 4.5 hr p.i. and then chased for 105 min. The molar ratios, presented in Table 8, are expressed relative to the amounts of NCVP·X synthesized at the same time. The value of the ratio Ca:NCVP·X, while never as low as unity, was found to increase progressively from about

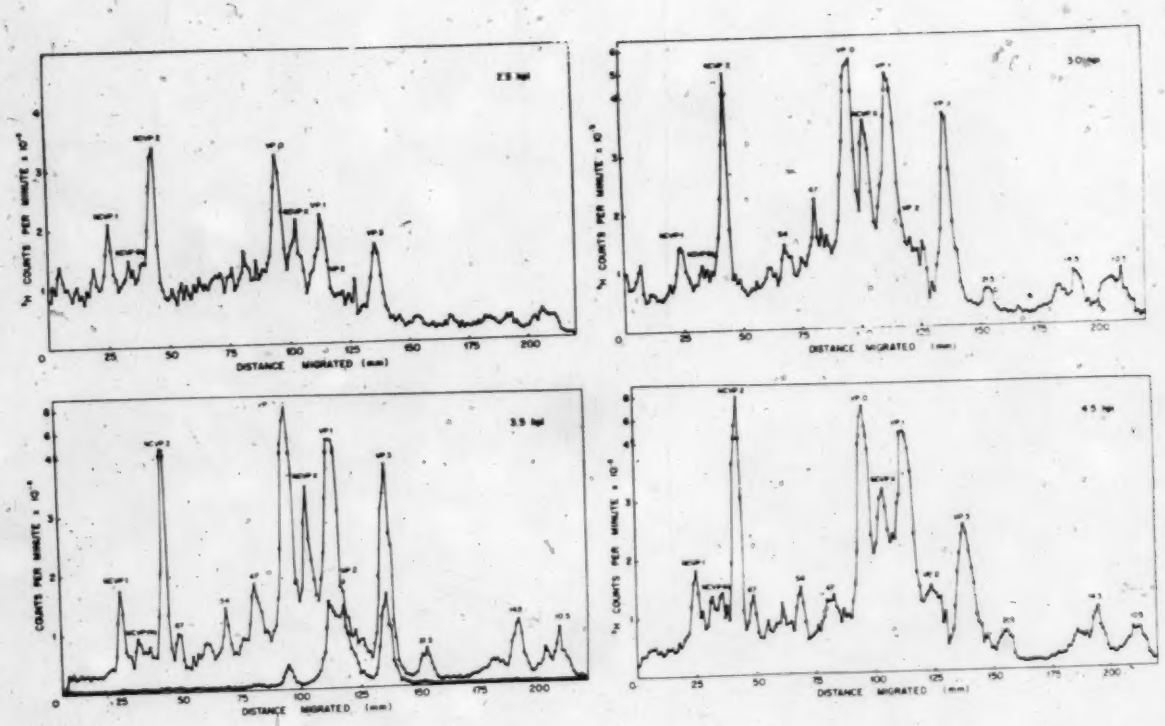


Figure 16. Electrophoresis in SDS-7.5% polyacrylamide gels of lysates of poliovirus-infected HeLa cells, pulse labelled with ^{3}H-amino acids for 15 min at 2.5, 3.0, 3.5 and 4.5 hr post-infection. Lysates were prepared after a chase period of 105 min. The closed circles in the third panel indicate the positions of the structural polypeptides of purified polio virions.

TABLE 8

Relative Molar Ratios of Poliovirus-Specific Polypeptides[1.]

Viral Polypeptide(s)	Molar ratio after pulse given at			
	2.5 hr p.i.	3.0 hr p.i.	3.5 hr p.i.	4.5 hr p.i.
EXP. 1				
Ca.[2.]	1.43	1.60	1.94	1.96
NCVP·X	1.00	1.00	1.00	1.00
N.Ca.[3.]	0.71	0.68	0.61	1.01
EXP. 2				
Ca	1.33	1.77	1.78	
NCVP·X	1.00	1.00	1.00	
N.Ca.	0.81	0.53	0.57	

1. Calculated from electropherograms obtained with lysates of cells pulse labeled for 15 min at the indicated times and chased for an additional 105 min.

2. The total amount of capsid protein synthesized (Ca) was calculated from $Ca = \frac{VP \cdot 0 + VP \cdot 1 + VP \cdot 3}{3}$

3. N.Ca = NCVP·2 + NCVP·4

1.4 at the earliest time at which such an analysis is feasible (2.5 hr p.i.) to about 2.0 late in the replicative cycle. Also listed in Table 8 are the values of the ratio N·Ca:NCVP·X at various times throughout the cycle. The value of this ratio was found never to exceed unity, and in fact was significantly less than unity in most analyses - a finding which is probably attributable to the instability of NCVP·4. Although the increase in the value of the ratio Ca:NCVP·X is not as marked as is the increase in the value of the comparable ratio [C(total):F] in the Mengo system, it seems clear that in the poliovirus system too the ratio of capsid:non-capsid present increases progressively as a function of time p.i.

Effect of amino acid analogues on synthesis of poliovirus proteins

An investigation of the effects of amino acid analogues on the synthesis of Mengo virus polypeptides (see Chapter III) revealed that, in the presence of these compounds, the amount of polyprotein Z (the translation product of the entire viral genome) relative to that of polypeptide A (the precursor of the capsid polypeptides) decreases progressively as a function of time p.i. - an observation compatible with our proposed model for control of translation. The data summarized in the preceeding section, which suggest that the poliovirus genome may be translated asymmetrically (at least late in the replicative cycle) made it seem worthwhile to carry out a similar study with this system. The same amino acid analogues - azetidine, canavanine, ethionine and ρ-fluorophenylalanine - were utilized to inhibit primary cleavages and to permit the detection of polyprotein NCVP·00.

Poliovirus-infected HeLa cells were pulse-labelled for 30 min in the presence of the amino acid analogues at 2.5, 3.0, 3.5, 4.0 and 4.5 hr p.i., lysed immediately thereafter, and the lysates subjected to electrophoresis in SDS-5% polyacrylamide gels. Representative electropherograms are presented in Fig. 17, from which it is quite obvious that the amount of NCVP·00 relative to that of NCVP·1 (the precursor of the capsid polypeptides) decreases progressively as a function of time p.i. The data were analyzed quantitatively and the results are summarized in Table 9, together with the molecular weights of the polypeptides. The molar ratio of NCVP·00 relative to NCVP·1 was found to decrease progressively, and very markedly, from a value of 1.68 at 2.5 hr p.i. to a value of 0.19 at 4.5 hr p.i. Also listed are the relative molar ratios of the two other high molecular weight polypeptides produced under these conditions - namely NCVP·0a and b, the lower and higher molecular weight components respectively in the peak identified as NCVP·0 in Fig. 17. Although the data suggest that there may be a progressive decrease in the relative molar ratio of NCVP·0b, and no consistent change in the value of the relative molar ratio of NCVP·0a, it is difficult to interpret the data concerning these two species since they (like polypeptides X and Y in the Mengo system) have not been identified.

Stability of the polyprotein NCVP·00

The results of the studies of the synthesis of viral proteins in the presence of amino acid analogues are compatible with our proposed model for the control of translation, in that the progressive decrease in the NCVP·00:NCVP·1 ratio which would be predicted from the model,

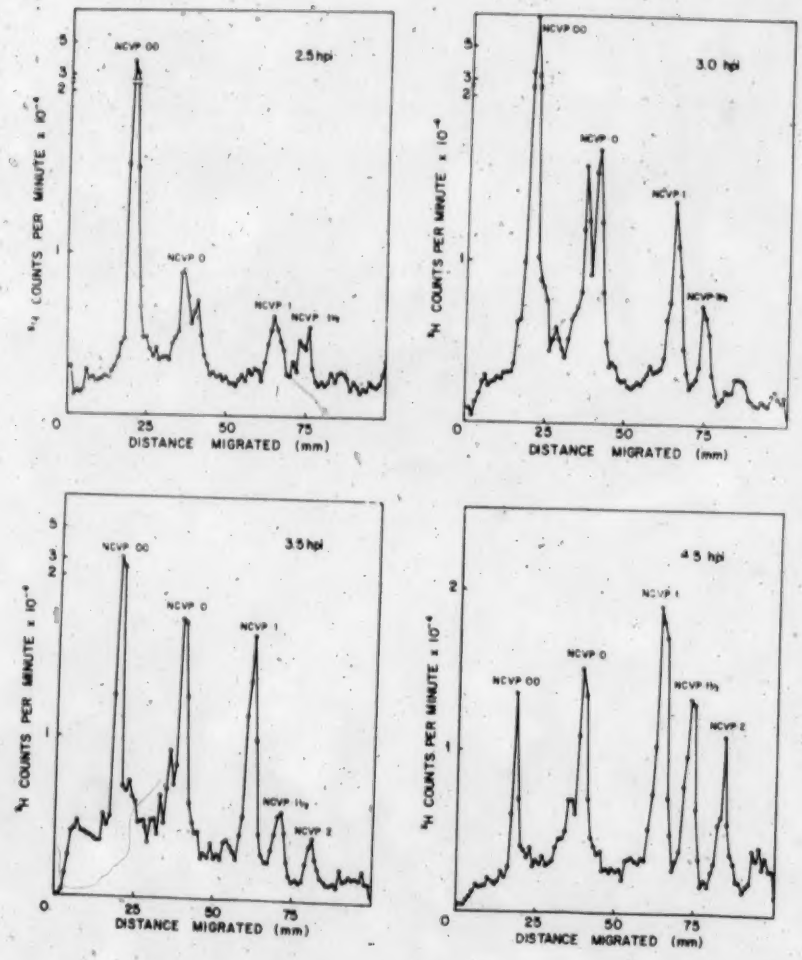


Figure 17. Electrophoresis in SDS-5% polyacrylamide gels of lysates of poliovirus-infected HeLa cells, pulse labelled for 30 min in the presence of amino acid analogues at 2.5, 3.0, 3.5 and 4.0 hrs p.i.

TABLE 9

Relative Molar Ratios of Poliovirus-Specific Polypeptides Produced in the Presence of Amino Acid Analogues

Polypeptide	Molecular weight x 10^{-3}	Molar ratio[1.] after pulse given at				
		2.5 hr p.i.	3.0 hr p.i.	3.5 hr p.i.	4.0 hr p.i.	4.5 hr p.i.
NCVP·00	229	1.68	1.41	0.68	0.38	0.19
NCVP·0b	158	0.58	0.42	0.30	0.24	0.15
NCVP·0a	145	0.60	0.79	0.69	0.60	0.46
NCVP·1	110	1.00	1.00	1.00	1.00	1.00

1. Values for NCVP·00, NCVP·0b and NCVP·0a are normalized with respect to NCVP·1.

was, in fact, observed.

However, there are other possible explanations, the most obvious one being that the decrease in the value of the ratio polyprotein: capsid precursor seen late in infection could be due to an increase, late in infection, of the activity of an enzyme (or enzymes) capable of degrading the polyprotein. For that reason, an experiment designed to examine the stability of NCVP·00 both early and late in infection was carried out. Replicate cultures of poliovirus-infected HeLa cells were pulse labelled in the presence of amino acid analogues at 2.5 and 4.5 hr p.i. Lysates were prepared after chase periods of 0, 10, 20, 30 and 60 min in the presence of amino acid analogues, and analzyed by electrophoresis in 5% polyacrylamide gels. The data are illustrated in Fig.18. A straight line was fitted to each set of points by the least squares method, and the half-life of NCVP·00 early and late in infection was estimated from the slopes of the two lines.

The data show that NCVP·00 is in fact degraded more rapidly late in infection, the estimated values for the half-life of NCVP·00 synthesized between 2.5 and 3.0 and between 4.5 and 5.0 hr p.i. being 74 and 42 min respectively. This finding suggests that either the amount or activity of enzymes capable of degrading the polyprotein increases late in the infectious cycle, and forces the conclusion that at least part of the observed decrease in the NCVP·00:NCVP·1 ratio (see Table 9) can be explained on the basis of an accelerated rate of breakdown of the polyprotein late in the cycle.

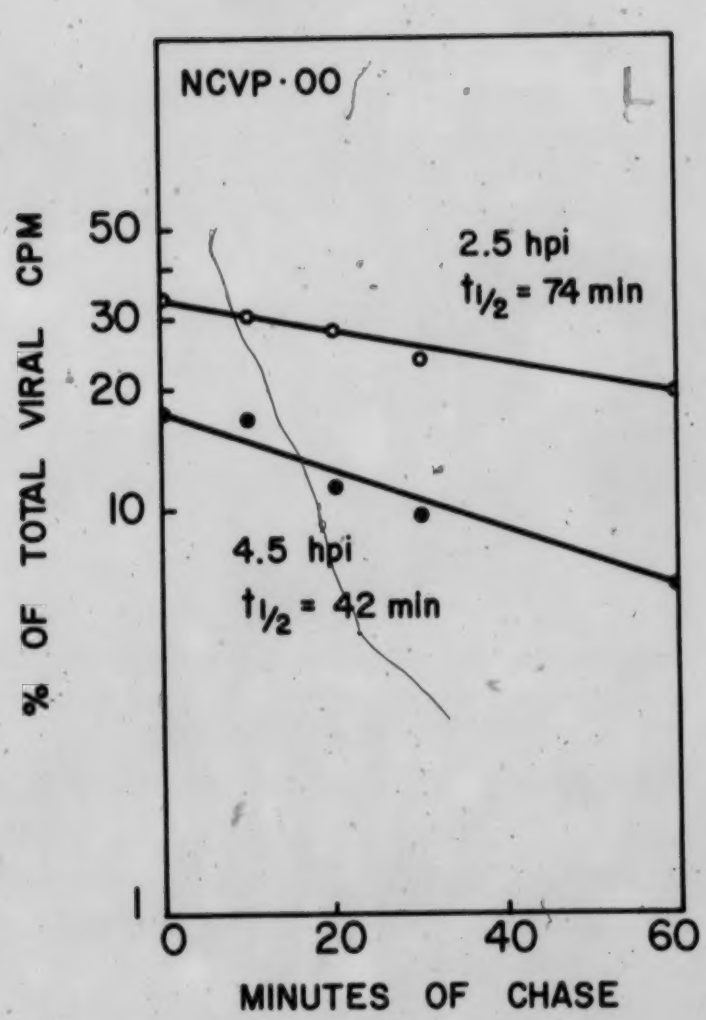


Figure 18. Stability of the poliovirus-specific polypeptide NCVP·00 synthesized during a 30 min period in the presence of amino acid analogues at 2.5 and 4.5 hr post-infection. In each case lysates were prepared and analyzed by electrophoresis in SDS-5% polyacrylamide gels after chase periods of 0, 10, 20, 30 and 60 min in the presence of amino acid analogues. $t_{1/2}$ = estimated half-life of polypeptide.

Discussion

The data presented in this chapter are in general agreement with those obtained from studies of the Mengo virus system. Approximately twice as much capsid as non-capsid protein is found in cell lysates prepared late in the infectious cycle, there is a progressive increase in the molar ratio of capsid:non-capsid protein as a function of time p.i., and - in the presence of amino acid analogues - a progressive decrease from early to late log phase of virus production in the value of the molar ratio of NCVP·00:NCVP·1. All these observations are compatible with the suggestion that the viral genome may be translated asymmetrically, and with the proposed model in which some viral specified protein acts as a specific termination factor capable of causing premature termination of translation at a site located near the midpoint of the viral RNA.

However, the poliovirus system is not as clean as is the Mengo system, and the data obtained from studies of the poliovirus system are subject to more uncertainties than are those obtained from the Mengo system. The large precursor proteins of the poliovirus system, for example, appear to be subject to ambiguous cleavages, and it seems likely that the relatively high value (1.4) of the ratio Ca:NCVP·X found early in the replicative cycle is a result of improper cleavage of NCVP·X. NCVP·5 (M.W. = 47,000) which maps near the center of the genome may well be an incorrectly cleaved NCVP·X. However, such ambiguous cleavages probably cannot explain the progressive increase observed in the ratio of capsid:non-capsid proteins, since the proportion of ambiguous cleavages is unlikely to increase with time.

Certain ambiguities are introduced by the presence of a number of minor polypeptides (NCVP·3-5 and 7-10) not found in the Mengo system. Their precise positions in the overall cleavage scheme are not known, and it is not clear whether they are products of ambiguous cleavages or degradation products of incorrectly cleaved viral polypeptides. Whatever the case, they were found in such small quantities that they were ignored in all analyses of the experimental data.

It could be argued that the results obtained with both the Mengo- and poliovirus systems reflect an accelerated and selective degradation of non-structural polypeptides late in the infectious cycle. The validity of the model proposed here depends on the assumption that the non-structural polypeptides - and most particularly the reference polypeptides F and NCVP·X - are relatively stable, or at least are not degraded at an accelerated rate late in the infectious cycle. The stability of the Mengovirus non-structural polypeptides (Lucas-Lenard, 1974; Paucha et al, 1974) appear to rule out this mechanism in the case of the Mengo system. The situation with respect to the poliovirus system is more uncertain in that no really definitive data regarding the stability of the non-structural polypeptides - particularly NCVP·X - are available. In this regard, it does seem clear from these studies that the Mengo non-capsid polypeptide E is considerably more stable than the corresponding poliovirus polypeptide NCVP·4, and it is interesting to note that the relative stabilities of these two polypeptides are consistent with earlier observations concerning the stabilities of polio and Mengo RNA replicases. Ehrenfeld et al (1970) found that when polio-infected cells were treated with cycloheximide, the viral RNA

replicase activity decayed rapidly (half life = 15 min), while Baltimore and Franklin (1963) reported that Mengo RNA synthesis continued at an undiminished rate for at least one hour after protein synthesis in infected L. cells was blocked by puromycin. Lundquist et al (1974) have presented evidence that NCVP·4 is the viral component of polio RNA polymerase, and Rosenberg et al (1972) reported earlier that a poly C-dependent RNA polymerase derived from the EMC RNA polymerase contains a polypeptide of molecular weight 57,000 - the same as that of polypeptide E, the EMC and Mengo equivalent of NCVP·4.

Taken at face value, the data obtained from the investigation of the synthesis of poliovirus polypeptides in the presence of amino acid analogues provides very convincing evidence of some kind of translational control in this system. However, the observation that the stability of NCVP·00 decreases late in the infectious cycle casts considerable doubt on the significance of the earlier observations.

Considered in toto, the data summarized in this chapter, while consistent with both the hypothesis that the synthesis of picornaviral proteins is subject to some kind of translational control and the proposed model, do not constitute the kind of convincing, supportive evidence that it was hoped they would provide.

V. VIRUS-SPECIFIC POLYSOMES

Introduction

The data presented in Chapter III suggest that during the replication of Mengo virus in L. cells, premature termination of translation at a site located near the midpoint of the viral RNA occurs with increasing frequency as infection proceeds. The studies described in this chapter were based on the premise that it should be possible to study this phenomenon more directly by examining the size and activity (in a cell-free system) of virus-specific polysomes from infected cells.

If only half of the viral mRNA is translated late in infection, the average size of polysomes should become progressively smaller during the mid to late log phase of virus production. Summers *et al* (1967) have reported that the size of poliovirus-specific polysomes isolated from infected HeLa cells decreases from about 380S to 200S late in infection, a finding they attributed to a decrease in the rate of translation. Since the individual viral polypeptides had not been identified clearly at that time, no quantitative estimate of their production could be made.

Roumiantzeff *et al* (1971a) have isolated membrane-bound polysomes from poliovirus-infected cells and have shown that these polysomes will synthesize *in vitro* a polypeptide of a size approximating that expected from uninterrupted translation of the entire viral genome (Roumiantzeff *et al*, 1971b). It was felt that the establishment of such a system from Mengovirus-infected L. cells might make it possible (i) to determine directly whether the termination phenomenon is real or only

apparent, and (ii) if real, to obtain some indication as to which viral protein may function as the termination factor.

While the work to be described in these two areas is fairly preliminary, it does provide a basis for more definitive studies.

Materials and Methods

Infection of cells

L. cells were grown to confluence in roller bottles as described in Chapter II. After decanting the growth medium, the cells were infected by adding 5 ml of virus suspension (in virus diluent; moi ≈ 100 pfu/cell) containing 5 μg Act.D/ml to each bottle. After 1 hour, during which time the bottles were rotated at 0.2 rpm, the inoculum was poured off, the cells rinsed with PBS to remove unattached virus, and 20 ml of BME containing 1% HS and 5 μg Act.D/ml were added. The roller bottle cultures were incubated at 37°C, and rotated at approximately 1 rpm.

To label the viral mRNA, ^{3}H-uridine (NEN) was added to the roller bottles at either 2 or 5 hours after infection to give a final concentration of isotope of either 5 or 10 μCi/ml.

To label nascent protein, the growth medium was replaced one hour before labelling by 10 ml amino acid deficient medium containing 1% HS, 25 mM HEPES and 5 μg Act.D/ml. One hour later this medium was replaced by the same medium supplemented with ^{14}C-amino acids (5 μCi/ml).

Preparation of cell extracts

Five or 6 hr p.i. cells were detached from the roller bottles by trypsinization, collected into 20 ml ice cold BME-5% HS, pelletted by low

speed centrifugation and washed sequentially with 20 ml volumes of ice cold PBS and RSB (reticulocyte standard buffer; 10 mM Tris, 10 mM KCl, 1.5 mM $MgCl_2$). The washed cells were then resuspended in 5 ml RSB and homogenized in an all glass Dounce homogenizer (Wheaton Glass Co., Brampton, Ont.) using a tight fitting pestle. The toncity of the homogenate was adjusted upwards at this point by the addition of sufficient 10X concentrated buffer to give final concentrations of 20 mM Tris, 5 mM $MgCl_2$, 100 mM KCl and 6 mM 2-ME (in subsequent sections the latter mixture is designated buffer 20-5-100-6). After removal of nuclei and unbroken cells by low speed centrifugation (3 min, 1500 rpm) the homogenate was separated into cytoplasmic supernatant (S_{20}) and cytoplasmic pellet (P_{20}) as described by Roumiantzeff et al (1971a) by centrifugation for 30 min at 20,000 xg (13 K rpm, JA 20 rotor, J21 Beckman centrifuge).

Sucrose density gradient analyses

Unless otherwise indicated, density gradient analyses of P_{20} and S_{20} fractions were carried out on 12 ml linear 15% to 45% (w/v) RNase-free sucrose gradients prepared in either RSB or a buffer composed of 20 mM Tris (pH 7.4), 5mM $MgCl_2$ and 100 mM KCl.

The cytoplasmic pellet (P_{20}) was resuspended in 1 ml of appropriate buffer, placed in an ice bath, and sodium deoxycholate (DOC) was added to a final concentration of 1%. After 5 min NP-40 was added to the same final concentration and a 500 µl aliquot of the suspension (containing about 5 A_{260} units) was layered immediately onto a gradient. About 700 µl (∿ 5 A_{260} units) of S_{20} was also analyzed.

The gradients were centrifuged at 36,000 rpm (120,000 xg) for

72 min (International B-60 ultracentrifuge, SB-283 rotor), after which they were fractionated using an ISCO Model D gradient fractionator equipped with a model UA-2 UV-monitor and a model 170 servographic recorder (Instrumentation Specialties Co. Inc., Lincoln, Nebraska).

In those cases where polysomes had been radiolabeled, 200 µl fractions were collected directly onto filter paper discs (Whatman no.3, 2.3 cm diameter), which were air dried before being washed sequentially with cold 10% TCA, cold 5% TCA, ethanol and acetone. Radioactivity measurements were made in a Beckman Liquid Scintillation Spectrometer (Model LS-230) using a toluene scintillation fluid.

Protein synthesis *in vitro*

Cell extracts were prepared as has been described except that after homogenization the final concentration of the homogenate was adjusted to 20 mM Tris, 5 mM $MgCl_2$, 80 mM KCl and 6 mM 2-ME (which will be referred to as buffer 20-5-80-6). Fraction P_{20}, obtained as described earlier, was resuspended in 1 ml of buffer 20-5-80-6, and 50 µl aliquots were mixed with equal volumes of fraction S_{20} and held in an ice bath. To each P_{20}-S_{20} mixture was added a 50 µl aliquot of a freshly prepared, chilled solution containing the remaining components of the reaction mixture, after which the samples were transferred immediately to a 37°C water bath. In addition to the components of buffer 20-5-80-6, the complete incubation mixture contained 1 mM ATP and 300 µM GTP (both from Raylo Chemicals, Edmonton, Alberta), 7.5 mM phospho-enol-pyruvate, 1.5 units of pyruvate kinase (both from Sigma), and 0.75 µCi ^{14}C-amino acid mixture (NEN).

For gel analysis, the reaction was terminated by adding 15 μl of a solution containing 10% SDS, 50% 2-ME and 10^{-2}M PMSF to the sample and immersing it in boiling water for 5 min.

To follow incorporation of ^{14}C-amino acids into acid-insoluble material, 20 to 50 μl aliquots were removed from the incubation mixtures and added to 2 ml of cold 10% TCA. After 15-30 min at 0°, the precipitates were collected by filtration through membrane filters (Gelman Instrument Co., Ann Arbor, Mich.; pore size = 0.45 μ). Radioactivity was measured in the usual way.

Polyacrylamide gel electrophoresis

Twelve cm long SDS-7.5% polyacrylamide gels were prepared as described earlier (Chapter III). They were pre-run for 1 hr at 8 mA/gel before use.

Samples which had been prepared as described above, were dialysed for several hours against 0.1M sodium phosphate buffer, pH 7.4, containing 2% SDS and 5% 2-ME before use. Due to the relatively large volume of the samples, polypeptides were run into the gels at a current of 2 mA/gel, after which electrophoresis was continued at 5 mA/gel until the bromphenol blue marker dye, which had been added to each sample, had migrated to within 1 cm of the bottom of the gel.

The procedures employed for staining and cutting the gels and for measuring radioactivity in the gel slices have been described (Chapter III).

Results

Isolation of polysomes from infected cells

Virtually all polysomes present in either infected or uninfected L. cells are found in the P_{20} fraction, indicating that these structures are firmly associated with intracellular membranes. Only a few small (slowly sedimenting) polysomes are found in the supernatant (S_{20}). These findings are illustrated by the data presented in Fig. 19, in which are shown the polysome profiles obtained with cytoplasmic pellets isolated from infected cells 6 hr after infection and from uninfected cells incubated for 6 hr in the presence or absence of actinomycin D. All P_{20} fractions were treated with DOC to release membrane-bound material before sedimentation. By blocking mRNA synthesis, actinomycin D reduces the number of polysomes found in uninfected cells (panel B) compared to the number present in uninfected cells which have not been exposed to the antibiotic (panel A). The number of polysomes present in cells 6 hr after infection with Mengo virus (panel C) is also reduced, an observation which is not surprising in view of the marked inhibition of cellular protein synthesis caused by this virus. A polysome profile representative of those obtained with S_{20} fractions prepared from either infected or uninfected cells is shown in panel D. In all cases, only a few small polysomes were found in this fraction.

Repeated analyses of P_{20} fractions from infected cells failed to detect any giant virus-specific polysomes comparable to those found by other investigators in poliovirus-infected HeLa cells (Summers et al, 1967; Roumiantzeff et al, 1971a). Addition of the potent ribonuclease inhibitor polyvinyl sulfate (Bernfeld, 1963) to the post-nuclear supernatant had no effect on polysome profiles obtained with P_{20}.

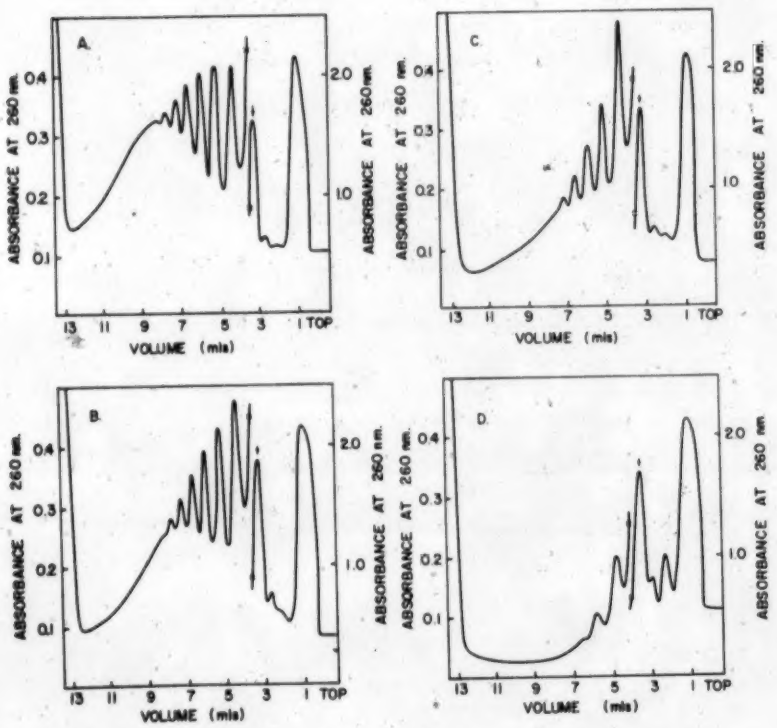


Figure 19. Gradient analysis of DOC-treated P_{20} fractions from L.cells. The position of intact, 80S ribosomes is indicated in each panel by an arrow. Note that the absorbance scale is amplified five-fold at the beginning of the polysome region. The direction of sedimentation is from right to left. Panel A: P_{20} fraction from uninfected cells; panel B: P_{20} fraction from mock-infected cells, prepared 6 hr after Act.D. treatment (mock-infection); panel C: P_{20} fraction from Mengo virus-infected cells, prepared 6 hr after infection; panel D: S_{20} fraction from Mengo virus-infected cells, prepared 6 hr p.i.

fractions. (Addition of the inhibitor to the whole cell homogenate resulted in nuclear lysis and release of the highly viscous DNA). The observation that trypsin, at concentrations commonly used in tissue culture manipulations, inhibits elongation of growing polypeptide chains in treated cells (Koch, 1974) made it seem unlikely that the absence of giant polysomes was due to ribosome run-off during the isolation procedures. This was verified by the finding that the addition of cycloheximide (up to 100 μg /ml, Noll and Burger, 1974), which also inhibits elongation, to the cultures just prior to harvest did not alter the polysome profiles obtained. Attempts to detect virus-specific polysomes by radioactive labelling of either the viral mRNA or nascent polypeptides were also unsuccessful; in both cases the label was distributed throughout the gradient. Evidently the polysomes are fragmented during the separation procedures, either at the stage where cells are swollen in hypotonic buffer (during which L. cells triple in volume, and without which the cells are not disrupted by homogenization) or at the stage of dissolution of supporting membranes by DOC. In any case, it was not possible to isolate intact viral polysomes by this method, and thus examination of their size as a function of time after infection was impossible.

Cell-free protein synthesis

Since virtually all polysomes are membrane-bound in infected L. cells, the P_{20} fraction was used, without DOC treatment or any further purification, to establish a relatively crude cell-free protein-synthesizing system. Gradient analysis of the P_{20} fraction without DOC treatment, which is presented in Fig. 20, shows that it contains a few

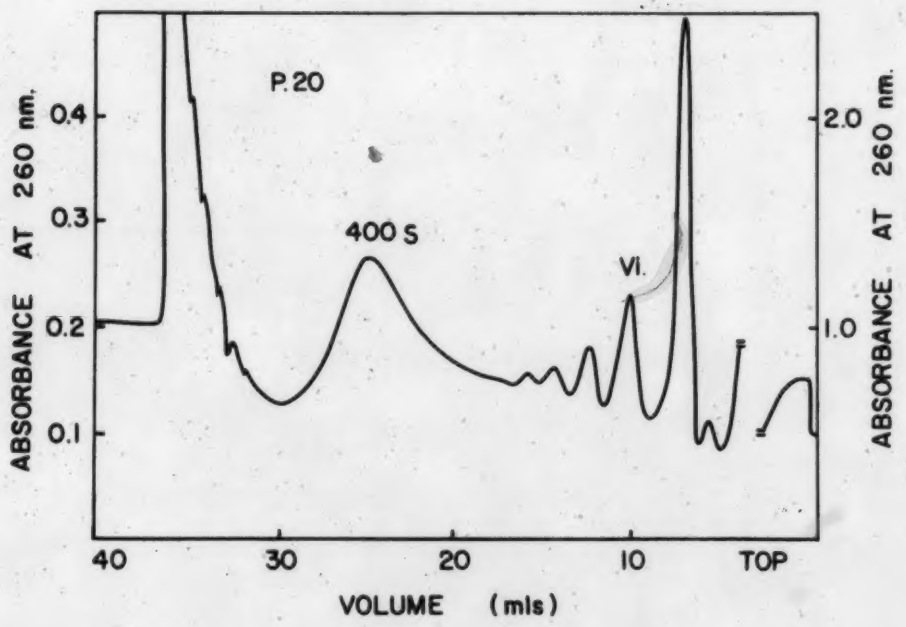


Figure 20. Gradient analysis of P_{20} fraction from Mengo virus-infected L.cells. The P_{20} fraction was prepared 6 hr p.i., resuspended in 20-5-100-6 buffer and layered without DOC treatment onto a 34-ml linear gradient of sucrose (15-45%) in buffer 20-5-100 on a 3 ml 60% sucrose cushion. Centrifugation was carried out at 25,000 rpm for 135 min in an SW27 rotor. Sedimentation is from right to left. Note the five-fold amplification of the absorbance scale at the top of the gradient. The position at which 150S Mengo virions band is indicated by Vi.

free polysomes (fractions 8 to 16) and a peak of membrane-bound material sedimenting at about 400 S (fractions 20 to 30). Large membrane aggregates are found at the bottom of the gradient on the 60% sucrose cushion.

Due to the great instability of the P_{20} fraction, it was usually used as soon as it was prepared. Overnight storage at 4°C either as a pellet or as a suspension in aqueous buffer resulted in the loss of about 50% of the activity. However, virtually no loss in activity was detectible if the pellet was resuspended in the presence of 20% glycerol and stored overnight at -20°C.

The optimal concentration of Mg^{++} for incorporation of amino acids into acid-insoluble products was found to be between 5 and 6 mM, as shown in Fig. 21. When incorporation was examined as a function of KCl concentration (in the presence of 5 mM $MgCl_2$) a curve with a broad maximum from about 70 to 85 mM was obtained (Fig. 22). On the basis of these observations, $MgCl_2$ and KCl were used at concentrations of 5 mM and 80 mM respectively in all subsequent experiments.

A time course of synthesis under conditions of optimum salt concentration is shown in Fig. 23. While very little incorporation was observed in the presence of the supernatant alone, substantial synthesis was found to occur in mixtures containing the resuspended pellet. Both the rate and extent of synthesis were shown to be increased when both the S_{20} and P_{20} fractions were present in the incubation mixture. Incorporation was found to reach a plateau at 60 min in both cases. The addition of 2×10^{-4} M TPCK to the complete system had no effect on incorporation.

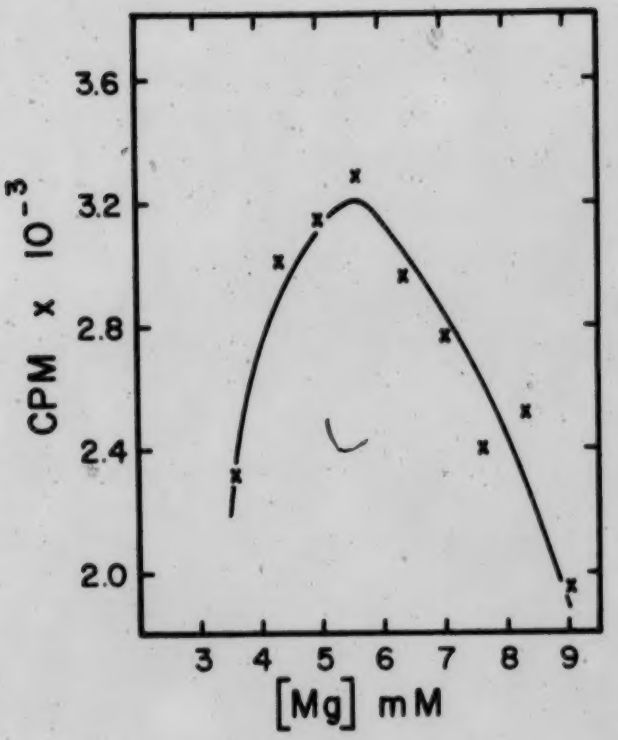


Figure 21. Effect of Mg^{++} concentration on Mengo virus polysome-directed protein synthesis in a cell-free system. Incubation mixtures of 120-µl were maintained at 37°C. One 50-µl aliquot was removed immediately after the addition of reaction mixture to the S_{20}-P_{20} mixture and a second 90 min thereafter. Samples were precipitated at 0° in 10% TCA, filtered, dried and counted as described. In all cases, the number of counts in the 'zero time' sample have been subtracted. The concentration of K^+ was 80mM.

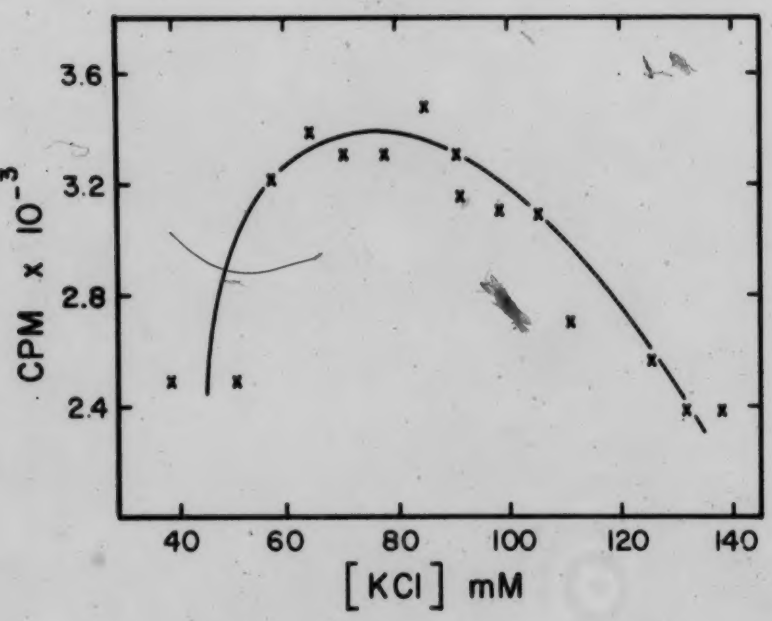


Figure 22. Effect of K^+ concentration on Mengo virus polysome-directed protein synthesis in a cell-free system. Incubation mixtures of 120-µl were maintained at 37°C. One 50-µl aliquot was removed immediately after the addition of reaction mixture to the S_{20}-P_{20} mixture and a second 90 min thereafter. Samples were precipitated, filtered, dried and counted as described. In all cases, the number of counts in the 'zero time' sample have been subtracted. The concentration of Mg^{++} was 5 mM.

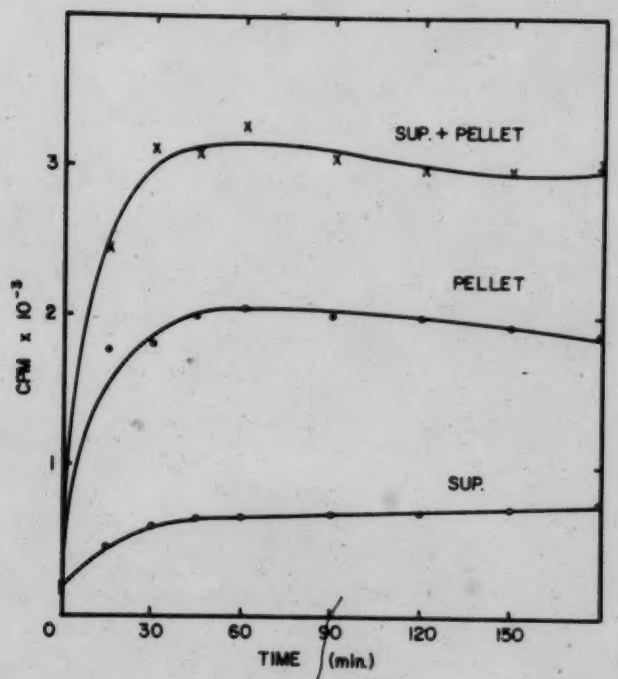


Figure 23. Kinetics of incorporation of amino acids in a cell-free protein synthesizing system. Incubation mixtures of 360 µl were maintained at 37°C. Duplicate 20 µl samples, removed at the times indicated, were precipitated, filtered, dried and counted as described. Each point represents the average value of the two samples.

Since this system appeared to be functional, the products were analyzed to determine whether large polypeptides or only small peptides were being synthesized.

Polyacrylamide gel analysis of products synthesized in cell-free system

The results of analyses on SDS-7.5% polyacrylamide gels of the products produced in the presence and absence of TPCK are shown in Fig. 24. From the left panel it is evident that some large polypeptides (MW 60,000 to 100,000) are synthesized by membrane-bound polysomes. In addition, a substantial amount of material whose molecular weight must be greater than 125,000 was found at the top of the gel. The fact that the addition of TPCK, which inhibits cleavage of the polyprotein precursor *in vivo* (Korant, 1972; Summers *et al*, 1972) doubles the amount of radioactivity in this high (>125,000) molecular weight fraction (Fig. 24, right panel) suggests that complete translation of the Mengo genome may take place in this system.

While the corresponding fractions derived from mock-infected cells were found to incorporate some radioactivity, gel analyses showed that very little protein of molecular weight >60,000 and none of molecular weight >125,000 is produced (data not shown).

Discussion

The crucial role played by cell membranes in the replication of poliovirus has been demonstrated repeatedly. Synthesis of viral proteins (Penman *et al*, 1964; Roumiantzeff *et al*, 1971b) and of viral RNA (Baltimore, 1964, 1969) takes place in membrane-associated complexes, and evidence has been presented to suggest that viral assembly is

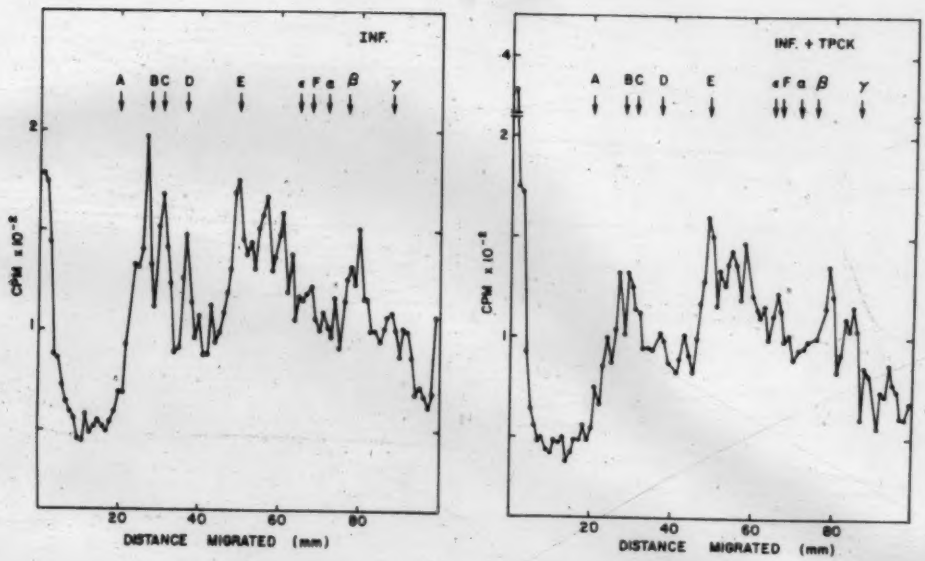


Figure 24. Analysis on SDS-7.5% polyacrylamide gels of the products synthesized in cell-free system. The positions of the Mengo virus-specific polypeptides, indicated by arrows, were determined by mixing aliquots of a lysate of infected cells with each sample prior to electrophoresis. Left panel: polypeptides produced without TPCK; right panel: polypeptides produced in the presence of 2 x 10^{-4} M TPCK.

initiated on cell membranes (Phillips, 1971). Thus it was not surprising to discover that both the synthesis of Mengoviral proteins and virus assembly are also associated with cell membranes. It was found that if Mengovirus-infected L. cells, which had been pulse-labelled with ^{3}H-amino acids from 5.5 to 6.0 hr p.i., were incubated in the absence of labeled amino acids for an additional hour, the proportion of the total cellular radioactivity present in the P_{20} fraction decreased by only some 12% (from 67% after a pulse to 55% after a pulse followed by a 1 hr chase).

In contrast to reported observations of the polio system, intact Mengo virus-specific polysomes could not be released from membranes. This may be due to a "destabilization" of the giant polysomes by dissolution of supporting membranes, but it is more likely that it is a result of swelling the cells in hypotonic buffer, a procedure which is required for efficient homogenization. The average cell volume triples during this procedure. It has been suggested (R. McElhaney, personal communication) that when such an extreme volume increase occurs, the endoplasmic reticulum may open out and form a part of the plasma membrane of the cell. Any linear structures bound to the endoplasmic reticulum would thus be subjected to extreme stress and could easily be sheared during homogenization.

Before resorting to Dounce homogenization, exhaustive efforts were made to recover polysomes from extracts prepared by treatment of infected cells with the non-ionic detergents Triton X-100 and NP·40. All were unsuccessful. It appeared that although these detergents damaged the cell membrane and caused cell lysis, they did not affect the

endoplasmic reticulum or the associated polysomes at all. As a result the entire complex remained attached to the nuclei (which are unaffected by these detergents) and pelleted with them. Sucrose gradient analyses of cytoplasmic extracts prepared by this procedure gave results very similar to those illustrated in Fig. 19, panel D, that is, the extracts contained a few small polysomes only. It is possible that undegraded virus-specific polysomes could be recovered by a procedure which combines detergent lysis and Dounce homogenization, particularly if these structures are broken down during the process of swelling the cells in hypotonic buffer. It would then be possible to examine polysome size throughout the infectious cycle, and to conduct definitive studies of viral polypeptide synthesis using the *in vitro* protein synthesizing system described here.

VI. THE 50S PARTICLE

Introduction

Wright and Cooper (1974) have reported that, in poliovirus-infected HeLa cells, a particle composed of equimolar amounts of the capsid polypeptides VP·0, 1 and 3 is associated with the smaller ribosomal subunit. During the course of comparable studies with EMC virus-infected cells, Medvedkina et al (1974) failed to find any ribosome-bound capsid proteins but did detect large amounts of the non-capsid polypeptide G associated with 80S ribosomes. Matthews et al (1973) had previously reported similar results with EMC virus-infected cells, and, in addition, had reported that most of the ribosome-bound label in HRV-1A-infected cells was found in a polypeptide of M.W. ≃ 76,000, which is identical to that of the non-capsid polypeptide D. Thus, it was of interest to determine whether any virus-specific polypeptides are bound to ribosomes in Mengo virus-infected L. cells.

During this study, a subviral particle sedimenting at about 50S in linear sucrose gradients was discovered. Since there is no completely convincing evidence for the occurrence of subviral particles in cells infected with cardioviruses, this finding was of considerable interest. The results of studies on the nature of this particle are presented here.

Materials and Methods

Infection of cells

Confluent monolayers of L. cells in roller bottles were infected with Mengo virus as described in Chapter V. At 4.5 to 5 hr after infection, the growth medium was replaced by amino acid deficient

medium and incubation was continued for 1 hr. At that time, the amino acid deficient medium was replaced by the same medium supplemented with a mixture of ^{14}C-amino acids (5 μCi/ml). After a 30 min labelling period, the cultures were rinsed with PBS and incubated with normal growth medium for an additional 30 to 60 min before being harvested. Additional experimental details are given in the figure legends.

Preparation of cell extracts

The procedures described in Chapter V for the preparation of cell extracts and for their separation into the S_{20} and P_{20} fractions were followed here with one modification. The washed cell pellet was resuspended for homogenization in 5 ml of a buffer containing 1mM Tris (pH 7.4), 1.5mM $MgCl_2$ and 1mM KCl rather than in RSB (10mM Tris, 1.5mM $MgCl_2$ and 10mM KCl). The extreme hypotonicity of this buffer resulted in extensive disruption of membrane-bound structures and gave maximum yields of 50S material.

Sucrose gradient analysis

All material having sedimentation coefficients of 15S or greater was recovered from the S_{20} fractions by centrifugation for 3 hrs at 45,000 rpm in a Beckman Type 50 rotor (g_{av} = 122,249) at 4°C before being analyzed by density gradient centrifugation. The opalescent pellets were redissolved gently in 1 ml of 20-5-100-6 buffer with the aid of a glass rod, and the samples were layered onto chilled 34 ml linear (15-45%) sucrose gradients in 20-5-100 buffer, prepared over a 3 ml cushion of 60% sucrose. The gradients were centrifuged for 12 hrs at 20,000 rpm in a Beckman SW27 rotor at 4°C after which they were separated into 1 ml fractions using the ISCO density gradient fraction-

ating system described in Chapter V and a Buchler fraction collector (Buchler Instruments Inc., Fort Lee, N.J.). A 200 µl aliquot of each fraction was added to a glass scintillation vial containing 1 ml 10% (v/v) acetic acid and 10 ml ScintiVerse scintillation fluid, and the radioactivity was measured in a Beckman Model LS-230 liquid scintillation spectrometer.

Recovery and analysis of material from sucrose density gradients

Appropriate gradient fractions were pooled, diluted with 20-5-100-6 buffer to reduce the sucrose concentration, and the material therein recovered by high speed centrifugation: 40,000 rpm for 18 hr (I.E.C. B-60 centrifuge, SB-283 rotor) for the 15, 40 and 50S material, and 25,000 rpm for 18 hr (Beckman L5-65 centrifuge, SW27 rotor) for those pooled fractions containing either 80S ribosomes or 150S virus particles.

For polyacrylamide gel electrophoresis, pellets were dissolved in 0.1 ml lysis mixture (see Chapter III) and dialyzed against 0.01 M phosphate buffer (pH 7.4) containing 2% SDS and 5% 2-ME before use.

Those samples which were analyzed by centrifugation in Cs_2SO_4 density gradients were dissolved in 2.5 ml volumes of 20-5-100 buffer, diluted with equal volumes of an aqueous solution of Cs_2SO_4 (ρ = 1.64 gms/ml) and centrifuged to equilibrium (35,000 rpm for 21 hr) in a Beckman SW 50.1 rotor. Fractions (6 drops) were collected from the bottom of the tubes onto filter paper discs which were air dried before being washed sequentially with cold 10% TCA, 5% TCA ethanol and acetone. Radioactivity measurements were made in a Beckman Scintillation Spectrometer (Model LS-230) using a toluene scintillation fluid.

Results

Identification of the 50S particle

In order to determine whether any Mengo virus-specific polypeptides become tightly associated with ribosomes during virus replication, infected L. cells were pulse-labelled for 30 min at 5.5 hr. p.i. and harvested after a chase period of 1 hr. The S_{20} fraction was prepared, and 1-ml aliquots were analyzed by velocity sedimentation using the large (34 ml) sucrose gradients which had been designed to separate ribosomes and large and small ribosomal subunits. The results of one such analysis are presented in Fig. 25. The pattern of absorbance at 260 nm (lower line) shows that virus particles, ribosomes and ribosomal subunits are resolved clearly in such gradients. Much of the radioactivity (upper line) was found to be associated with virions which sediment at 150S, but in addition a well-defined peak of labelled material (sed.coeff. ≈ 50S) was found at a position between those occupied by the ribosomal subunits. A small amount of radioactive protein was found to be associated with intact (80S) ribosomes, and a substantial amount was present at the top of the gradient.

Before any further studies could be carried out, it was necessary to recover these particles from the entire cytoplasmic supernatant (S_{20} fraction). The procedure chosen was to centrifuge the S_{20} fraction as described in the Materials and Methods section of this chapter in order to pellet all sedimentable material. This pellet was then resuspended and separated into its various components by centrifugation through 34 ml sucrose density gradients. The profile of radioactivity obtained from such a gradient analysis is shown in Fig. 26. As a

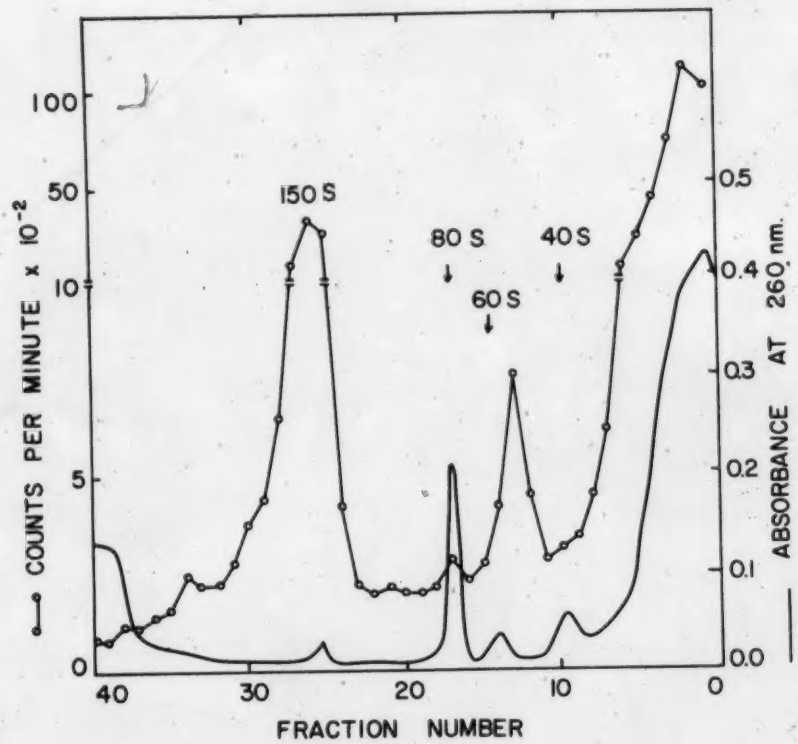


Figure 25. Gradient analysis of fraction S_{20} from L.cells, prepared 6 hr. after infection with Mengo virus. Sedimentation is from right to left.

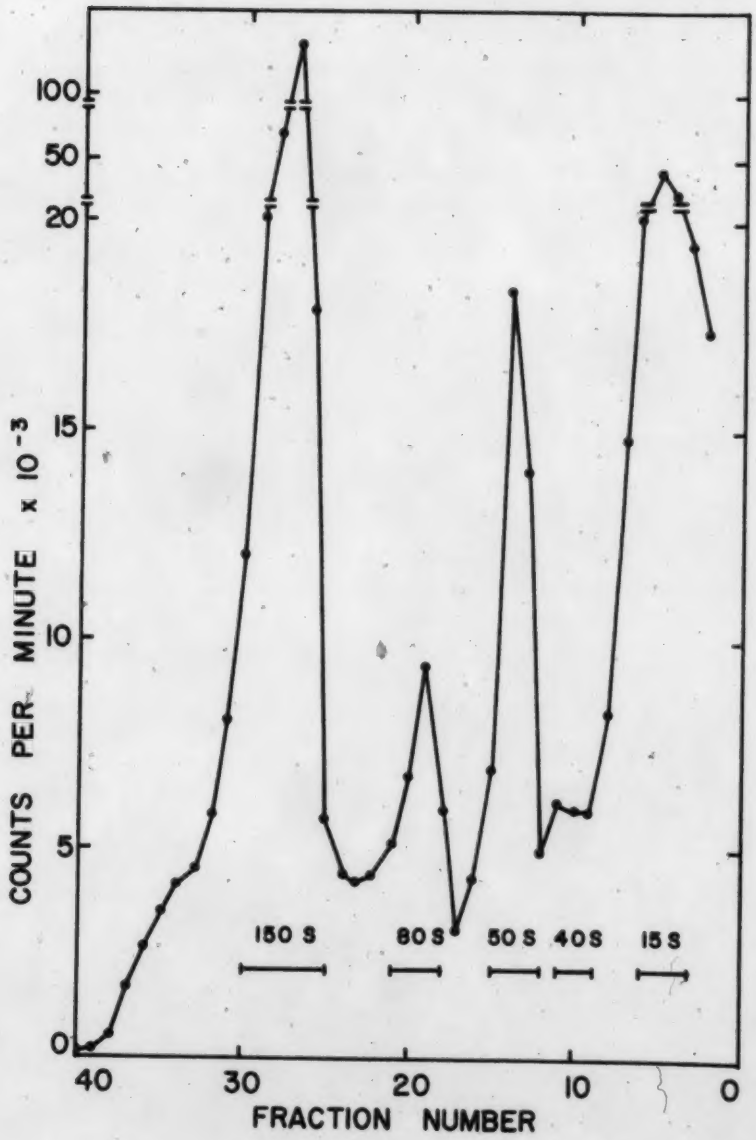


Figure 26. Gradient analysis of sedimentable components of fraction S_{20}. Sedimentation is from right to left. The horizontal bars indicate the fractions pooled.

result of the pelleting procedure, the amount of radioactivity remaining at the top of the gradient is greatly decreased, thus making clearly visible a peak of material having a sed.coeff. of about 15S. In addition to the radioactivity present in 150S virions, in 80S ribosomes and in 50S particles, a small amount was also found to be associated with the small ribosomal subunit.

In order to determine which viral polypeptides were present in these fractions, each was subjected to SDS-polyacrylamide gel electrophoretic analysis.

Polyacrylamide gel electrophoresis of gradient fractions

Fractions obtained after sucrose density gradient centrifugation of the sedimentable components of fraction S_{20} were pooled as indicated by the horizontal bars in Fig. 26, and the radioactive material in each pool was recovered by centrifugation as described in the Materials and Methods section of this chapter. The pellets were resuspended in 'lysis mixture' and analyzed on SDS-10% polyacrylamide gels. Representative electropherograms are shown in Fig. 27. It is clear that both the 15S and 50S particles are composed of equimolar amounts of the capsid polypeptides, ε, α and γ. In the 150S virions, α and γ are present in equimolar amounts, but little ε remains since it is cleaved to β and δ during the assembly process. In contrast, the 40S and 80S ribosomal structures contain little if any radioactivity in proteins which are demonstrably virus-specific. When compared with gels prepared from the corresponding fractions of uninfected cells (data not shown), the 40S fraction appears to be enriched in polypeptide G. All other peaks are also found in ribosomes of uninfected cells and thus may

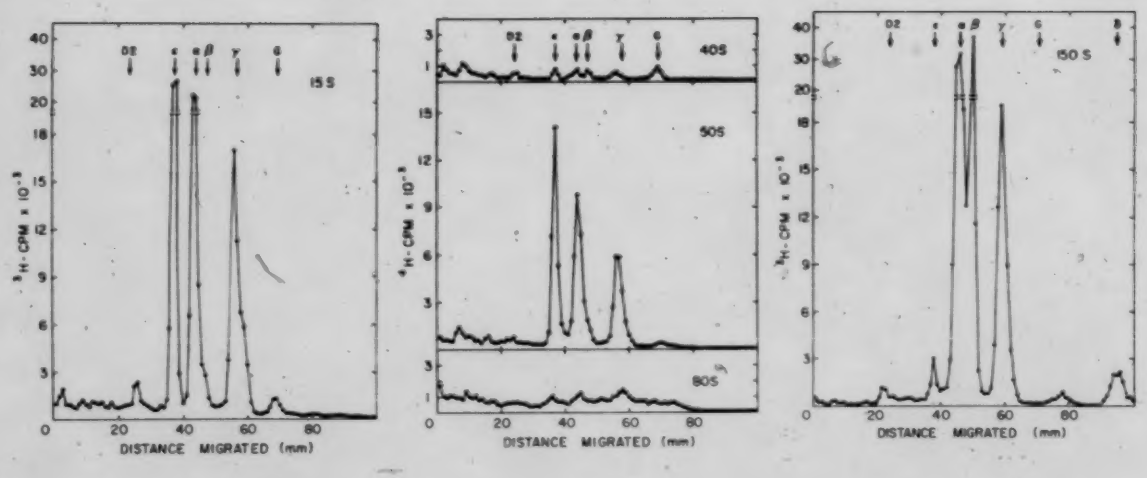


Figure 27. Analysis on SDS-7.5% polyacrylamide gels of sedimentable components of S_{20}. Positions of viral polypeptides, here indicated by arrows, were determined by mixing aliquots of a lysate of infected cells with each sample before electrophoresis.

represent residual incorporation into ribosomal proteins. Between 70 and 80% of the radioactivity present in each of the pooled gradient fractions was recovered from the corresponding gel.

From these data, it was concluded that ribosomes isolated from Mengo virus-infected L. cells by this procedure contain no bound virus-specific polypeptides, although a trace amount of polypeptide G appears to be attached to the smaller ribosomal subunit.

The finding that both 15S and 50S particles are composed of equimolar amounts of the capsid proteins ε, α and γ was an intriguing one. The 15S particle, whose sedimentation coefficient was not determined accurately in these studies, is probably identical to the 14S pentamer first found by McGregor et al (1975), and whose polypeptide composition is $(\varepsilon\alpha\gamma)_5$. The composition of the 50S particle indicates that it may be either an intermediate in the assembly process between the pentamer and the virion, or a "dead-end" aggregate of (εαγ) protomers. Kinetic studies were carried out in an attempt to distinguish between these two possibilities.

Kinetic studies of Mengoviral assembly

Mengo virus-infected L. cells were pulse-labelled for 15 min at 5.75 hr p.i. and cytoplasmic supernatants (S_{20}) were prepared after chase periods of 30, 90 or 210 min. The supernatants were concentrated by centrifugation and the pellets analyzed on sucrose density gradients as before. The number of counts in each fraction was plotted as a percentage of total counts recovered from the gradient as shown in Fig. 28. The data show that the radioactivity in both the 15S and 50S fractions is transferred nearly quantitatively into the virus peak, from which it

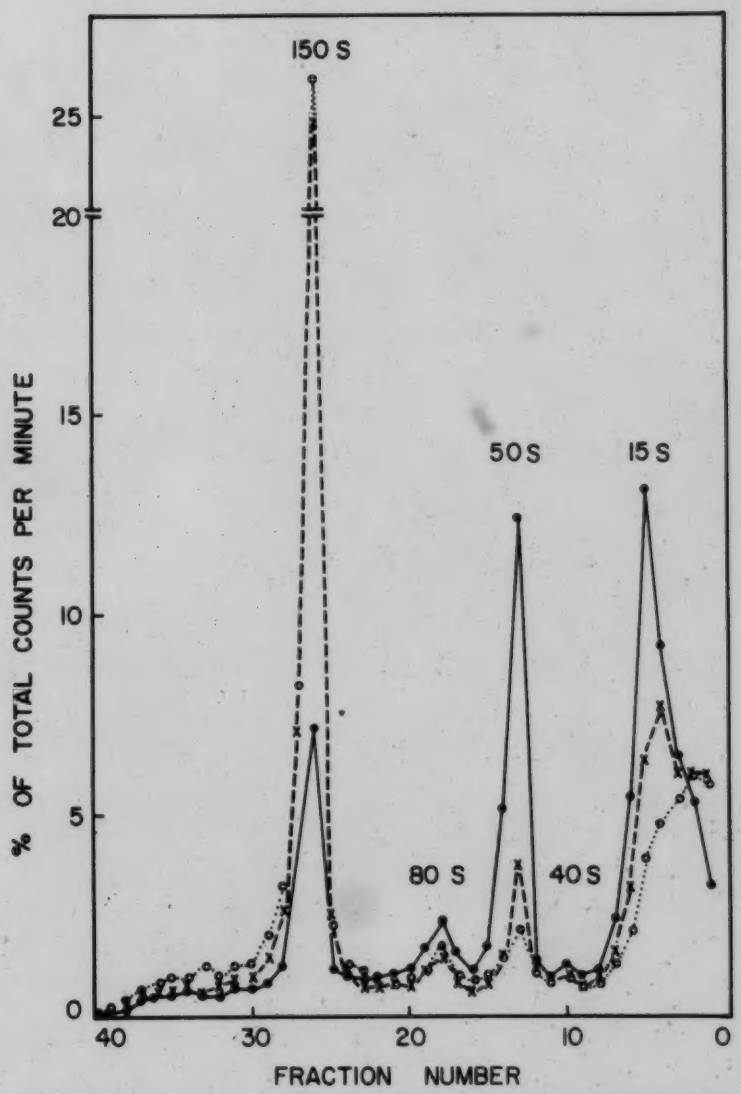


Figure 28. Kinetic studies of Mengo viral assembly. Gradient analysis of concentrated S_{20} fractions prepared after a 15 min pulse with ^{14}C-amino acids administered at 5.75 hr. p.i. followed by a chase period of an additional 30 min (●——●), 90 min (x --- x), or 120 min (o ... o).

seems safe to conclude that both are viral precursors, and that 50S particle is not merely a "dead-end" aggregate of ($\varepsilon\alpha\gamma$) protomers.

A 50S particle could serve as a true assembly intermediate in one of two ways: either it could be the product of association of the 35S viral RNA and a single capsid pentamer and thus serve as a "seed" for further assembly, or it may represent the cardiovirus analogue of the 73S procapsid found in cells infected with enteroviruses. In an attempt to distinguish between these two alternatives, experiments were carried out to determine whether or not the 50S particle contains RNA.

RNA in the 50S particle

Infected L. cells were labelled with ^{14}C-amino acids from 5 to 5.5 hr after infection. Ten minutes before the end of a 30 min chase period, ^{3}H-uridine was added to the cultures. The cells were harvested at 6 hr. after infection, fractionated, and the sedimentable components of fraction S_{20} isolated as described earlier. Aliquots of the latter were incubated at 37°C for 20 min in the presence or absence of 20 μg/ml RNase before being subjected to velocity centrifugation in sucrose density gradients. The distribution of amino acid (^{14}C) and uridine (^{3}H) label in the gradients is shown in Fig. 29. It is clear that the amount of 50S material is unaffected by RNase treatment, which decreases both the absorbance at 260 nm (not shown) and the ^{14}C-radioactivity in the ribosome peaks by more than 30%. Virtually no labelled uridine was detected in the 50S region in either case. These data suggest that the 50S particle does not contain RNA.

The conclusion that the 50S particle is composed solely of protein was substantiated by the results of studies of its behavior during

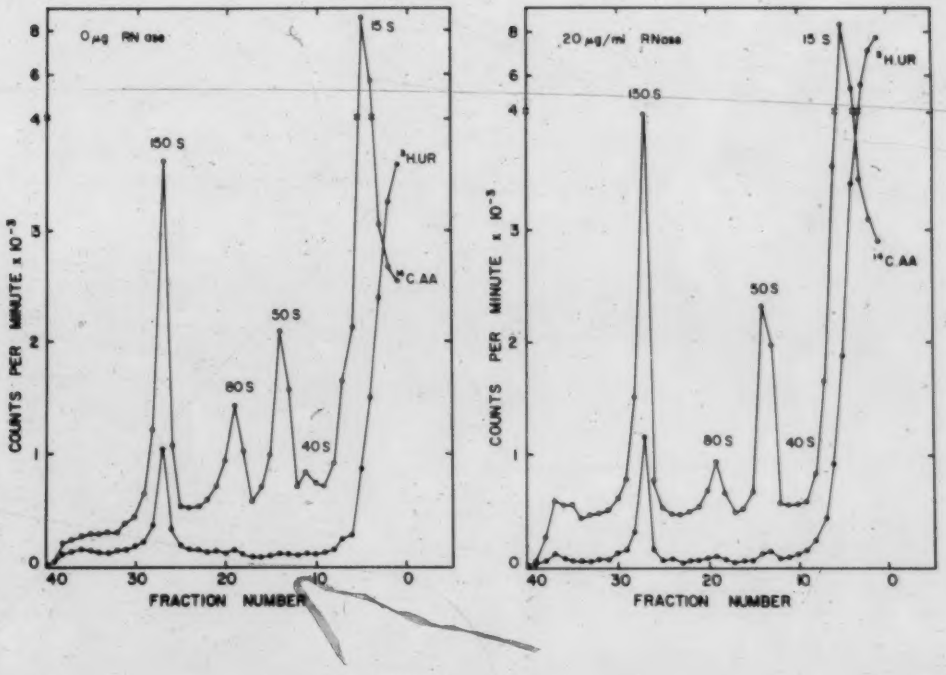


Figure 29. Effect of RNase on the 50S particle. Gradient analysis of concentrated S_{20} fractions following incubation (20',37°C) in the presence or absence of RNase. (●——●) ^{3}H-Uridine distribution, (○——○) ^{14}C-amino acid distribution.

equilibrium centrifugation in Cs_2SO_4 density gradients. The results illustrated in Fig. 30 show that when ^{14}C-amino acid labelled 50S particles were centrifuged to equilibrium in a Cs_2SO_4 gradient, virtually all the radioactivity was found at a buoyant density substantially lower than that of intact virions (which were run in a parallel gradient), but very close to that which would be expected of a particle containing protein only. No radioactivity was found near that part of the gradient where viral RNA would band (fractions 4-6) and where the 50S particle would be expected to band were it composed predominantly of RNA. A significant fraction of the radiolabel was found at a buoyant density lower than that characteristic of pure proteins, which suggests that the 50S particles may contain membrane fragments.

Discussion

Ribosome-bound viral polypeptides

The data presented here suggest that there is little or no association of virus-specific polypeptides with ribosomes in Mengo virus-infected L. cells, the only evidence for an association being the detection of trace amounts of polypeptide G in the sucrose gradient fractions containing the smaller ribosomal subunit. These observations differ from those published by several other groups. Medvedkina et al (1974) reported that relatively large amounts of polypeptide G are associated with intact (80S) ribosomes in EMC virus-infected Krebs II cells, and Matthews et al (1973) reported a similar finding in the case of EMC virus-infected HeLa cells. The latter group also found what they assumed to be two virus-specific polypeptides of M.W. = 76,000 and

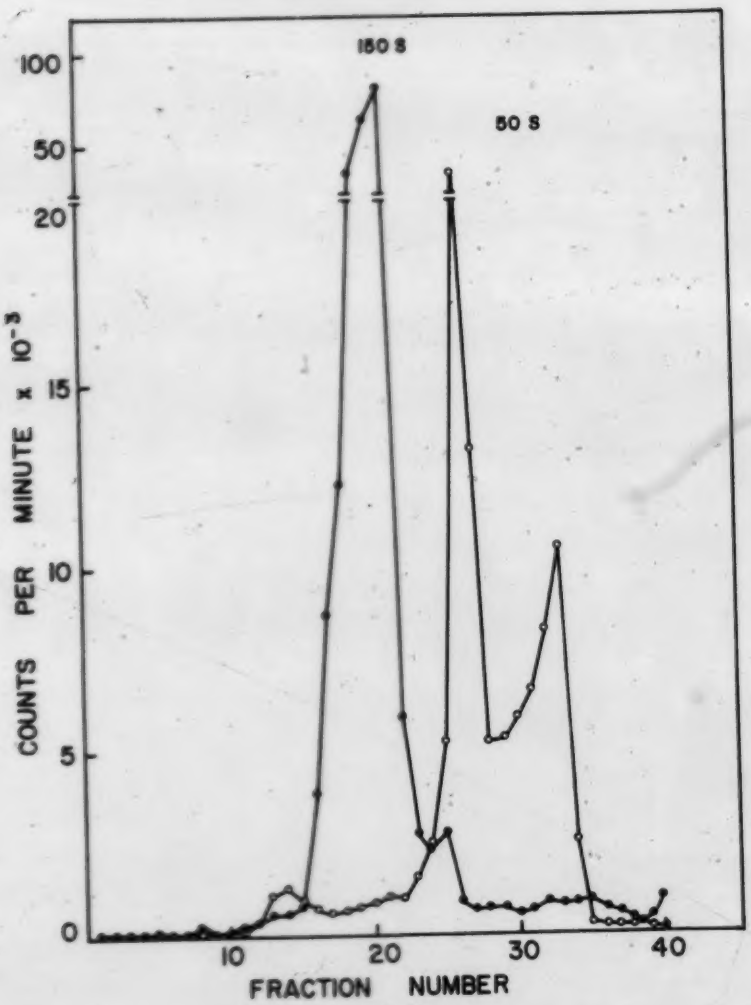


Figure 30. Equilibrium centrifugation in Cs_2SO_4 density gradients. The 150S and 50S fractions, pooled from gradients as indicated in Fig. 26, were centrifuged in separate gradients and are superimposed here for comparison. The density increases from right to left in the figure; 150S virions band at a density of 1.34 gms/ml. (●——●) 150S virions fraction; (o——o) 50S particle fraction.

38,000 associated with ribosomes isolated from HRV-1A-infected HeLa cells. Wright and Cooper (1974) have reported that the 45S ribosomal subunits isolated from poliovirus-infected HeLa cells carry the capsid polypeptides VP·0, 1 and 3.

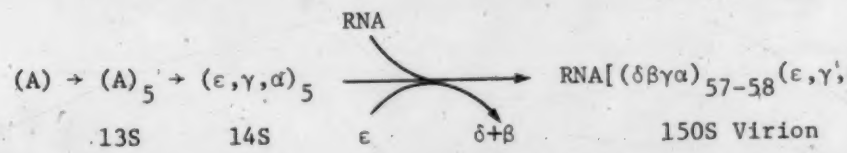
It is not easy to reconcile our observations with those of these other workers, - particularly those concerning the association of polypeptide G with intact ribosomes. However, the apparent association of capsid polypeptides with the 40-45S ribosomal subunit may well be a reflection of incomplete resolution of ribosomal subunits from a structure comparable to the 50S particle described here. It is pertinent to point out that the patterns of labelled proteins found in the 40S and 80S fractions from mock-infected cells were essentially identical to those found in the same fractions from infected cells (the only significant difference being the presence of small amounts of polypeptide G in the 40S fraction from infected cells), and that the molecular weights of these ribosomal proteins are close to those of the larger (M.W. > 20,000)"viral polypeptides" found on ribosomes by other workers.

The 50S particle

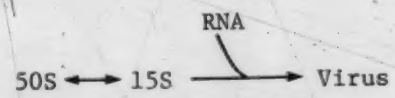
The assembly of the cardioviruses, unlike that of the enteroviruses, appears to proceed in a concerted fashion without the formation of stable intermediate subviral particles. In only one instance have such particles been observed. Prather and Taylor (1975) have reported the detection of 80S and 125S particles in lysates of Mengo virus-infected Madin-Darby bovine kidney (MDBK) cells. MDBK cells are considered to be non-permissive for Mengo virus, since the viral output is only about

5 pfu/cell. Since neither the polypeptide composition of these particles nor the kinetics of their formation have been established, their possible role as intermediates in the assembly process remains to be determined.

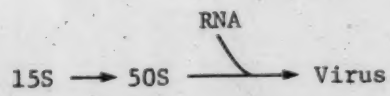
Based on available evidence, McGregor et al (1975) have proposed the following scheme for the assembly of EMC virions.



Although the kinetic studies presented here suggest that the 50S particle is an intermediate in the assembly process, its precise position in the scheme is as yet unknown. It is not possible from the experiments described here to determine whether the 50S particle is merely a reversible aggregation of protomers (or pentamers), in which case the assembly process could be visualized as follows,



or whether it is a procapsid-like structure and a true intermediate in the pathway of viral assembly. Diagrammatically, the scheme would then be represented thus,



It is difficult to rationalize the low value of its sedimentation coefficient if in fact the 50S particle plays a role in the assembly of Mengo virus analogous to that of the 73S procapsid in the enterovirus assembly pathway. However, the Cs_2SO_4 equilibrium centrifugation studies reported here suggest that the 50S may be membrane associated.

The presence of membrane fragments could conceivably retard its migration through sucrose solutions and thus result in an anomalous value for the sedimentation coefficient.

The well-characterized 73S procapsid and 125S provirion are believed to be the products of successive steps in the assembly of enteroviruses. Whether similar structures are produced during the assembly of cardioviruses and have escaped detection due to their inherent instability, or whether viruses in the two groups are assembled by different mechanisms has been a matter for debate for some time. Studies of attachment and uncoating have shown that the entire cardiovirus capsid structure is less stable than that of the enteroviruses (Joklik and Darnell, 1961; Hall and Rueckert, 1971). Further characterization of the 50S particle may shed some light on this question.

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